DEVELOPMENT, CHARACTERIZATION AND INITIAL ANALYSES OF CYSTEINE-RICH INTESTINAL PROTEIN TRANSGENIC MICE

By

BARBARA A. DAVIS

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To Paul, for his love and support and most importantly for being my best friend.

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ABBREVIATIONS

bp	basepair
BSA	bovine serum albumin
CRIP	cysteine-rich intestinal protein
rhCRIP	recombinant human cysteine-rich intestinal protein
CRIPTG	cysteine-rich intestinal protein transgene/transgenic
cDNA	complimentary deoxyribonucleic acid
Dex	dexamethasone
ddH$_2$O	distilled, deionized water
DMEM	Dulbecco's modification of Eagle's medium
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
ECL™	enhanced chemiluminescence
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
GALT	gut-associated lymphoid tissue
FITC	fluroscein isothyocyanate
ICBR	Interdisciplinary Center for Biotechnology Research
IEC-6	intestinal epithelial cells
IgG	immunoglobulin G
i.p.	intraperitoneal
kb	kilobase
kDa	kilodalton
LPS	lipopolysaccharide
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPF	specific pathogen free
TBS	tris-buffered saline
UAB-NTMDF	University of Alabama/Birmingham - National Transgenic Mouse Development Facility
UV	ultraviolet

Abstract of Dissertation Presented to the Graduate School
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DEVELOPMENT, CHARACTERIZATION AND INITIAL ANALYSES OF CYSTEINE-RICH INTESTINAL PROTEIN TRANSGENIC MICE

By

Barbara A. Davis

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Major Department: Food Science and Human Nutrition

Cysteine-rich intestinal protein (CRIP) is a developmentally-regulated, LIM-only protein which binds zinc. Although CRIP was identified a decade ago, the biological function of this metalloprotein remains unknown. In an attempt to provide additional information about this protein and its zinc binding properties, transgenic mice were created which overexpress rat CRIP (rCRIP) in a tissue-specific fashion. This project involved sequencing the rCRIP structural gene, which revealed five exons and four introns. In addition to providing information about the genomic organization of CRIP, sequencing this gene provided the necessary information for production of the rCRIP transgene (rCRIPTG) construct. The polymerase chain reaction (PCR) was used to produce a 4.5 kb transgene construct which contained the CRIP promoter and structural gene plus 91 bp of vector sequence.

Following production of the rCRIPTG construct, it was purified and sent to the University of Alabama-Birmingham, National Transgenic Mouse Development Facility (UAB-NTMDF) for microinjection into mouse oocytes. DNA samples from mice developed from the microinjected oocytes were then analyzed by PCR in our lab for the presence of the transgene. Mice identified as being transgenic were used to establish a CRIP transgenic mouse colony. Characterization of these mice included determining transgene copy number, CRIP RNA and protein expression in various tissues, histologic examination of the intestine and immunohistochemical localization of CRIP in the intestine, thymus, spleen, lymph node and peritoneal macrophage.

CRIP was shown to be localized in regions of lymphoid tissue where cells tend to undergo proliferation and/or maturation. Flow cytometric analysis of thymic lymphocyte populations showed that transgenic mice have a significantly greater proportion of $CD4^+/CD8^+$ lymphocytes compared to their non-transgenic littermates. Collectively, these data support the theory that CRIP may be involved in immune function, perhaps in the regulation of immune cell proliferation or maturation. Transgenic mice which overexpress CRIP will provide a novel model for future studies examining the biological function of this metalloprotein.

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

Cysteine-rich intestinal protein (CRIP) is a LIM protein with a high affinity for zinc. The LIM motif, with the consensus sequence Cys-Xaa$_2$-Cys-Xaa$_{17\text{-}19}$-His-Xaa$_2$-Cys-Xaa$_2$-Cys-Xaa$_2$Cys-Xaa$_{7\text{-}11}$-(Cys)-Xaa$_3$-Cys (Michelson et al., 1993), forms a double zinc finger which binds two zinc atoms per motif *in vitro*.

CRIP was identified a decade ago during the screening of an intestinal cDNA library for clones which were developmentally regulated (Birkenmeier and Gordon, 1986). The developmental regulation of CRIP has subsequently been confirmed, and it has been postulated that CRIP may play a role in the intestinal maturation process. It has also been suggested that CRIP may be involved in zinc absorption, although more recent evidence supports a role for this protein in processes associated with cellular proliferation, differentiation or turnover, especially in lymphoid tissue. A definitive biologic role for CRIP has not yet been identified.

The goal of this dissertation project was to develop a model for studying CRIP overexpression in a whole animal system using transgenic technology. The establishment of a transgenic mouse line for the purpose of increasing CRIP expression is important because there are currently no known methods by which CRIP expression can be significantly increased. Although CRIP is a zinc-containing protein, its expression cannot be induced by zinc. This is in contrast to another small cysteine-rich zinc-binding protein, metallothionein, which is transcriptionally regulated by zinc.

Several tools of molecular biology have been used for this project, demonstrating that these techniques can be an important component of nutrition research and can enhance our understanding of nutrient metabolism at a molecular level. First, the CRIP structural gene was sequenced. This demonstrated several putative transcription regulatory sites, including a site for the general transcription factor Sp-1, which strengthened the hypothesis that CRIP plays an important housekeeping function, as this transcription factor is known to regulate expression of several housekeeping genes. Sequencing also provided valuable information for the design of a CRIP transgene construct for the production of transgenic mice. The production of transgenic mice which overexpress CRIP provided the opportunity to study the consequences of CRIP overproduction in otherwise normal animals. This overexpression model will be useful for future studies aimed at defining the function(s) of CRIP.

It is of nutritional interest to further study CRIP because of its ability to bind zinc. Zinc-finger proteins are known to have essential physiological roles and, because of their large number, probably contribute significantly to dietary zinc requirements. Expanding our knowledge of the function of these zinc-containing proteins will enhance our understanding of the nutritional importance of zinc and may also uncover a biomarker for zinc status. Studying CRIP is also of nutritional relevance because of its potential role in development and maintenance of the intestine and possibly immune cells.

Objectives

The objectives of this research are to:

1. Provide additional information about the genomic organization of CRIP by determining the sequence of the rat CRIP structural gene.

2. Examine the tissue distribution of CRIP mRNA in mice and to use immunohistochemical techniques to determine whether CRIP protein corresponds to mRNA levels.

3. Develop and perform initial characterization of transgenic mice containing extra copies of the rCRIP gene for investigation of the physiologic response of rCRIP overproduction in an effort to provide additional information about the biological function of this protein.

4. Determine whether increased CRIP levels in thymus will alter the type or maturity of cells in this lymphoid tissue.

Hypotheses

1. The tissue distribution of CRIP expression is similar between mice and rats.

2. CRIP overexpression in transgenic mice will not be a lethal mutation and will provide a useful model for further study of CRIP function.

3. CRIP will be highly expressed in lymph nodes similar to other immune tissue.

4. CRIP plays a role in the proliferation or differentiation of immune cells.

Literature Review

Cysteine-Rich Intestinal Protein

Cysteine-rich intestinal protein (CRIP) was first identified in 1986 by Birkenmeier and Gordon during the screening of a rat small intestinal cDNA library for mRNA whose expression was developmentally influenced. Since its initial identification in small intestine, CRIP mRNA has been detected in lung, colon, stomach, spleen, thymus, placenta, heart, ovary, uterus, skin, adrenal, muscle, brain, peritoneal macrophage and peripheral blood mononuclear cells (Birkenmeier and Gordon, 1986; Levenson et al., 1993; Fernandes et al., 1996 and Hallquist et al., 1996). Expression is highest in the intestine and immune cells (Hallquist et al., 1996). Using immunohistochemical techniques, CRIP has recently been localized to the Paneth cells as well as the enterocytes at the villus tip of the rat small intestine (Fernandes et al., 1996). Paneth cells are a terminally differentiated cell type which reside in the crypt region of the intestine. They are one of four cell lineages derived from the same multipotent stem cells that the villus enterocyte arises from, and are the only cell type which migrate downward from the crypt-villus junction (Gordon et al., 1992). Paneth cells are thought to be involved in the intestinal mucosal barrier as they have been shown to secrete the antimicrobial defensins (Selsted et al., 1992) and cryptdins (Jones and Bevins, 1992) as well as lysozyme (Erlandsen et al., 1974) and tumor necrosis factor (Keshav et al., 1990). Interestingly, significant quantities of zinc have also been located within granules of rat Paneth cells (Dinsdale, 1984). The distinct tissue distribution of CRIP may prove to be a key component in elucidating the biological role of this protein. For this reason, a tissue distribution of CRIP expression in mice was performed as part of this dissertation project.

The developmental regulation of CRIP expression has been further studied (Levenson et al., 1993; Needleman et al., 1993). Levenson et al. (1993) showed an increase in CRIP mRNA levels from d 19 of gestation to postnatal d 21, when expression reaches adult levels. They also showed that treatment with the glucocorticoid, dexamethasone (Dex), on postnatal d 1 and 2 doubled CRIP expression on postnatal d 3. The ability of glucocorticoids to alter CRIP expression was confirmed by Needleman and colleagues (1993) who showed that the normal rise in CRIP expression during the third postnatal week of life was depressed in adrenalectomized rat pups but was not prevented, as CRIP mRNA levels were able to reach levels of sham-operated pups by postnatal d 26.

In addition, they demonstrated that Dex administration on postnatal d 10 and 16 significantly increased jejunal CRIP mRNA levels, but injection on d 18 did not cause a significant elevation in CRIP mRNA . Data from these studies suggest that CRIP expression is sensitive to glucocorticoids in an age-dependent fashion. This is of interest because in rats, intestinal maturation, demonstrated by a rise in sucrase and isomaltase activity, occurs during the same period as CRIP mRNA levels rise in the intestine. It has therefore been proposed that CRIP may function as a mediator of glucocorticoid-regulated intestinal development (Needleman et al., 1993). Arguing against this role, however, is the abundance of CRIP mRNA in the Paneth cells of the intestine and in immune tissues. There are no data documenting whether CRIP expression is under developmental regulation in immune cells/tissues.

Involvement of CRIP and LIM Proteins in Immune Function

The presence of relatively high amounts of CRIP mRNA in immune cells provokes the hypothesis that this protein is somehow involved in immune function. Several pieces of evidence support this theory, including data obtained from our laboratory as well as information gathered about other LIM proteins. A recent report from our laboratory (Hallquist et al., 1996) supports the notion that CRIP expression is regulated in response to an immune challenge induced by lipopolysaccharide (LPS). In this study, rats were injected i.p. with LPS at 6, 12, 24 and 72 h prior to sacrifice. CRIP mRNA and/or protein levels were determined at each timepoint in the intestine, peritoneal macrophages, peripheral blood mononuclear cells, spleen, thymus, brain, liver and plasma. A rise in CRIP mRNA in PBMC, peritoneal macrophage and intestine late in the LPS-induced acute-phase response (48 h) was identified. Plasma CRIP protein was shown to decrease following LPS injection. However, more recent data from our laboratory do not support the presence of CRIP protein in plasma (unpublished observations). Elevated levels of

CRIP relatively late in the immune response may suggest that CRIP is involved in tissue repair. Alternatively, the rise in CRIP levels late in the immune response could mean that it plays a role in down-regulating this response.

CRIP mRNA expression is highest in the small intestine and peripheral blood mononuclear cells. However, thymus and spleen cells express abundant CRIP message as well. A clue to determining the function of this protein could be found by examining the functions of these lymphoid tissues. The thymus is a central or primary lymphoid tissue involved in the proliferation and maturation of T lymphocytes (Janeway and Travers, 1996). Lymphocytes produced in the bone marrow travel to the thymus where they differentiate into two T cell subsets, T helper cells and T cytotoxic/suppressor cells, which are distinguishable by cell surface receptors. Mature T cells then undergo thymic selection, a process where T cells specific for "self" antigens are destroyed. Proliferation, maturation and selection processes are dependent upon interactions between the developing cells and the thymic microenvironment. Proliferation of immature thymocytes (immature T cells) occurs in the outer cortical layer of the thymus whereas thymocytes undergoing thymic selection are located in the inner cortical layer. Mature thymocytes are present in the medulla as are macrophages and Hassal's corpuscles which are thought to be the site of cell destruction within the thymus (Burkitt et al., 1993).

The relative abundance of CRIP in the thymus suggests that this protein may be involved in regulation of T lymphocyte proliferation, maturation or destruction. Since these processes tend to be compartmentalized within the thymus, immunohistochemical localization using a CRIP antibody may provide insight as to which of these functions it is involved in. In addition, comparison of localization between normal and CRIP transgenic mice will help confirm the specificity of our antibody and enable more definitive localization of CRIP protein. Flow cytometric analysis of the lymphocyte populations within the thymus of normal and transgenic mice could provide information about whether CRIP has any influence on the development of cells within this organ. For example, if CRIP is involved in proliferation of lymphocytes within the thymus, it would be expected that immature thymocyte numbers might be altered in CRIP transgenic mice compared to their non-transgenic littermates. Following similar rationale, there may be an altered number of mature lymphocytes (i.e., T cells displaying specific cell surface receptors) in transgenic vs. non-transgenic mice if CRIP functions in a differentiation/maturation capacity.

The spleen, a secondary lymphoid tissue, is involved in destruction of aged erythrocytes via phagocytosis by macrophages in addition to production of immunological responses against blood-borne antigens. Similar to the thymus, the architecture of the spleen is partitioned into areas of distinct cell type and function. Lymphoid aggregations occur in areas known as the white pulp and removal of aged and defective blood cells and particulate matter takes place within the red pulp. Lymphocytes, both B and T cells, comprise between 5 and 20% of the spleen mass (Burkitt et al., 1993). In an attempt to gain insight into the function of CRIP in this organ, immunohistochemical techniques will be employed to determine where CRIP is localized within this organ.

Gut-associated lymphoid tissue (GALT) is considered to be a secondary lymphoid tissue. Considerable numbers of lymphocytes are present within the epithelium and lamina propria of the intestine and could contribute to the high levels of CRIP found in this tissue. Although GALT is formed during fetal life, germinal centers, consisting of lymphocytes do not develop until exposure to antigens after birth (Shanahan, 1994). This could be of significance because of the known developmental regulation of intestinal CRIP expression. In rats and mice, maximal post-natal CRIP levels occur around the same time as weaning. It is interesting to speculate that this elevation in CRIP expression occurs due to increased antigen exposure at weaning. Also of note is the presence of CRIP in mononuclear cells isolated from human milk (Lanningham-Foster and Cousins, unpublished data). The expression of CRIP in mouse milk cells is currently being investigated and correlated with its occurrence in the mouse gut.

There is precedence for the involvement of LIM proteins in the differentiation of lymphocytes. The LIM-homeodomain protein LH-2 was shown to be expressed in B- and T-lymphoid cell lines (Xu et al., 1993). Its expression pattern in B-cell lines was greatest in those lines representing early stages of differentiation, whereas no distinct correlation with differentiation stage was noted for LH-2 in T-cell lines. Based on its expression pattern and structural characteristics (i.e., member of LIM-homeodomain subclass), LH-2 is proposed to be a transcriptional regulatory protein involved in controlling cell differentiation of lymphocytes.

As noted above, our lab has documented high levels of CRIP expression in macrophages (Hallquist et al., 1996). The abundance of macrophages in lymphoid tissue is presumably responsible for a considerable amount of the CRIP found in this tissue and in peripheral blood mononuclear cells. Therefore, the role of various agents in regulating the expression of CRIP in a human monocyte/macrophage cell line (RAW 246.7) has been investigated (Cousins and Clark, unpublished data). LPS treatment (100 μg/mL) significantly depressed CRIP mRNA in RAW cells beginning at 6 h and continuing through 24 h. IFNγ treatment enhanced CRIP mRNA expression consistently by 6 and 24 h after addition to culture medium. This suggests that other effects elicited by endotoxin treatment of RAW cells are responsible for decreasing CRIP mRNA and that macrophage activation itself does not result in a decline in CRIP expression.

Available evidence strongly supports a role for CRIP in immune cells. However, it is uncertain whether CRIP is involved in maintenance of specific immune cells or whether it is involved in activation/suppression of cells during an immune response. The constitutive, high expression of CRIP in some lymphoid tissue implies that CRIP probably performs a "housekeeping" function such as controlling/regulating proliferation, differentiation or turnover of immune cells. The regulation of CRIP by cytokines suggests that CRIP may also be involved in other facets of the immune response as well.

Involvement of CRIP in Transmucosal Zinc Transport

The method by which zinc absorption occurs has not been established. Several lines of evidence, including the strong induction of metallothionein (MT) by zinc, suggest a role for this protein in regulation of zinc absorption (Cousins, 1989). However, the protein(s) responsible for transport of zinc across the intestinal epithelium remain to be identified. CRIP was proposed to function as an intestinal zinc carrier (Hempe and Cousins, 1991 and 1992). CRIP was shown to bind ^{65}Zn during transmucosal transport in an in vivo isolated intestinal loop procedure (Hempe and Cousins, 1991). A greater percentage of ^{65}Zn was associated with CRIP when luminal zinc was kept below the saturation concentration for transport compared with higher luminal zinc concentrations. This is in agreement with kinetic data which suggest zinc absorption includes both passive diffusion and saturable carrier-mediated components. In addition, CRIP was shown to bind progressively less of ^{65}Zn taken up from the lumen as the luminal zinc concentration increased from 5-300 μM (below to above zinc saturation concentration) whereas metallothionein associated ^{65}Zn increased with increased luminal zinc concentrations. Thus, a hypothetical model for zinc absorption was presented in which CRIP and MT play inverse roles in order to maintain zinc homeostasis (Hempe and Cousins, 1992). In this model, CRIP functions as a diffusible transport protein that binds zinc in the intestinal epithelial cell for subsequent transport across the cell to the basolateral membrane.

Elevated levels of MT, induced for example by high dietary zinc intake, would compete with CRIP for zinc binding thereby moderating zinc absorption.

Although CRIP is found in other tissues, levels are higher in the intestine than most other tissues (Hallquist et al., 1996). Furthermore, the developmental rise in intestinal CRIP levels demonstrated by Levenson et al. (1993) is consistent with the increase in carrier-mediated zinc absorption observed in the neonate (Ghishan and Sobo, 1983). However, several lines of evidence have surfaced which argue against the biological function of CRIP as a zinc trafficking protein. Recently, Fleet et al. (1993) demonstrated a reduction in CRIP mRNA levels and a concomitant rise in transepithelial zinc transport in Caco-2 cells following treatment with $1\alpha,25(OH)_2D_3$. Although this does challenge the theory that CRIP plays a primary role in zinc absorption, the method used by Fleet and colleagues (1993) to measure changes in CRIP expression may also be questioned. Since CRIP mRNA in the Caco-2 cell line is not easily detectable by Northern analysis, reverse-transcriptase polymerase chain reaction (RT-PCR) was used to detect relative changes in CRIP expression. Southern analysis was then used for quantitation of the amplified products. Although amplification of a control transcript, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used, this method can still only be considered a semi-quantitative approach, since differences in amplification efficiency may exist between primer pairs and product sizes differ by 25%. Whereas this method may be sufficient to make statements about very large differences between experimental groups, the sensitivity of this method is not adequate for detection of small changes in transcript levels of relatively low abundance. The theory that CRIP is involved in intestinal zinc trafficking has also been challenged because CRIP protein levels in the intestine do not appear to be affected by changes in zinc status (Hempe and Cousins, 1991) and intestinal CRIP mRNA levels are not altered by dietary zinc (Levenson et al., 1994b), whereas zinc absorption is sensitive to fluctuations in dietary zinc content and varies with respect to zinc status. Collectively, the data produced thus far suggest CRIP is probably not involved in zinc absorption.

Based upon current knowledge of its localization, developmental regulation and structural characteristics (i.e., LIM motif), several functions for CRIP may be postulated. Its localization in Paneth cells, immune cells and lymphoid tissue, coupled with its regulation by lipopolysaccharide, suggests a role for CRIP in immune function (Hallquist et al., 1996). This may be an indirect effect or suggestive of a role in cellular differentiation or proliferation/turnover of immune cells. The up-regulation of CRIP in the neonatal intestine suggests a role in intestinal development. Increased CRIP expression coincides with weaning, a period of marked alteration in dietary intake and subsequent changes in intestinal function. Therefore, CRIP could be involved in intestinal cell differentiation. Alternatively, because weaning results in an alteration in intestinal microflora, CRIP may increase as an adaptive response to a changing intestinal microbial environment. Clearly, additional investigation is required to ascertain whether any of these proposed biological functions for CRIP are correct. In addition, examining the biological roles of other LIM proteins may aid in our understanding of the function of this double zinc finger domain, and in turn, may provide insight into the biological function of CRIP as well.

LIM Motif and LIM Proteins

CRIP is a member of the LIM family of proteins. The cysteine-rich, double zinc finger motif which typifies members of this protein family was originally identified in the deduced protein products of the genes *lin-11*(Freyd et al., 1990), *isl-1* (Karlsson et al., 1990) and *mec-3* (Way and Chalfie, 1988). The LIM protein family, which has grown from three to more than fifty members in the six years since identification of its first members, is named for the first letter of those genes originally identified with this characteristic motif.

Because the proteins comprising this family are so diverse, members have been characterized into groups according to the presence or absence of other functional domains (Dawid et al., 1995). The original three LIM proteins belong to the LIM-homeodomain subclass. Members of this class are transcription factors involved in cell fate determination and differentiation (Sanchez-Garcia and Rabbitts, 1994; Dawid et al., 1995). Interestingly, the LIM domains of these proteins are thought to interfere with binding between the homeodomain and DNA (Sanchez-Garcia et al., 1993) which is in contrast to classical zinc finger domains which play an important role in DNA binding (Rhodes and Klug, 1993; Klug and Schwabe, 1995). Most members of this group have 2 LIM domains. A second group of LIM proteins, known as LIM-only proteins, contain 1-5 tandem LIM domains but lack a homeodomain. CRIP, rhombotin 1 and 2 (Ttg-1 and 2), cysteine-rich protein (CRP), muscle LIM protein (MLP) and PINCH are members of this subclass. Rhombotin 1 and 2 are oncogenes shown to be essential for erythroid development in Ttg-2 null-mutation mice (Warren et al., 1994) and capable of causing T-cell leukemia in Ttg-1 or Ttg-2 transgenic mice (McGuire et al., 1992; Wadman et al., 1994). MLP is also important in development as it has been shown to play an essential regulatory role in myogenesis by promoting differentiation (Arber et al., 1994). These data support a role for LIM-only proteins in the regulation of cell proliferation and differentiation. The abundance of CRIP in tissues which undergo periods of active proliferation and differentiation lends credence to the theory that CRIP follows other LIM-only proteins and plays a regulatory role in these processes.

Proteins such as zyxin and paxillin are members of a third class of LIM-containing proteins. Members of this class contain 1-4 LIM motifs in their C-terminus. A fourth class of proteins contain LIM motifs in their N-terminus in addition to other motifs upstream of the LIM domain. LIMK, which contains a kinase domain in addition to two LIM domains, is a member of this family (Sanchez-Garcia and Rabbitts, 1994).

Although LIM proteins are diverse and may contain other functional domains, their LIM motif is highly conserved. Each domain coordinates 2 zinc ions which causes folding into the zinc finger array, with two residues separating the fingers (Michelsen et al., 1993, Kosa et al., 1994). The actual function of the LIM domain itself is not known, but has been the subject of some recent investigations (Crawford et al., 1992; Feuerstein et al., 1994; Arber and Caroni, 1996). The existence of CRIP and the rhombotins, proteins which have little other unique sequence aside from their LIM domains, has led to the theory that these domains function in protein-protein interactions. The available evidence indicates that this is probably so. For example, Feuerstein and colleagues (1994) demonstrated that the LIM-only protein, CRP, can homodimerize *in vivo* and *in vitro*. By disrupting the double zinc finger domain via cysteine-to-serine substitutions, these investigators also demonstrated that the zinc-coordinated LIM motif was essential for homodimerization to occur. These findings provided evidence that LIM proteins are capable of protein interactions through their LIM domain and suggest that other LIM proteins may interact in a similar fashion, possibly with each other.

Sadler and colleagues (1992) showed that the two LIM domain proteins, zyxin and cCRP, interact in vitro and are co-localized in cytoskeleton of fibroblasts, indicating that the LIM domain may serve as a protein-binding interface. A recent report demonstrating that a single LIM motif is capable of selectivity in its binding suggests that these domains may selectively target proteins to specific interactions (Arber and Caroni, 1996). The LIM motif may be capable of binding to other binding domains as well. Wadman and colleagues (1994) demonstrated that RBTN2/Ttg-2 associates with the basic helix-loop-helix (bHLH) motif of several leukemogenic proteins, resulting in promotion of T cell leukemia. The association among the bHLH proteins TAL1, TAL2 and LYL1 and Ttg-2 appears to be specific. Co-expression of TAL1 and Ttg-2 in a two-hybrid system resulted in an increase of reporter gene activity, but substituting CRIP, CRP or zyxin for Ttg-2 did not generate a detectable increase in reporter gene activity. These data support a function for the LIM domain in allowing for specific protein-protein interactions.

A transgenic mouse model was recently developed to study the LIM protein rhombotin or Ttg-1. Overexpression of rhombotin, using a thymus-specific promoter, resulted in T cell leukemia/lymphoma, with tumor incidence being proportional to transgene expression (McGuire et al., 1992). This transgenic mouse model demonstrated that aberrant expression of the transcription factor, rhombotin-1, results in tumorigenesis due to overproduction of immature lymphocytes. This represents an important model for studying the etiology of T cell acute lymphoblastic leukemias and demonstrates the usefulness of transgenic models for investigation of protein function.

Transgenic Technology

Since the first reports of transgenic technology, which demonstrated the basic techniques of introducing foreign DNA into mammalian cells, the field of transgenics has rapidly progressed. Earliest accounts of transgenic technology reported the successful introduction of foreign DNA into cultured mammalian cells (Wigler et al., 1977, Minson et al., 1978, Maitland and McDougall 1977). Subsequently transfection of cultured cell lines with foreign DNA constructs has become quite common. Such transfection assays are used to determine sequences responsible for regulation of gene expression (Karin and

Haltgreve 1984, Levenson et al., 1993, Stuart et al., 1988, 1989), as well as sequences required for normal gene expression and to examine the effect of foreign proteins on cell function and differentiation (Knapp and Kopchick 1994).

Evidence that foreign DNA could be stably integrated into the germ line of animals was published shortly after the initial cell transfection reports. The establishment of the first line of transgenic mice was accomplished by retroviral infection of mouse embryos with foreign DNA (Jaenisch 1976, 1977). A major disadvantage of retrovirus infection involves problems with inconsistent expression of the transduced gene in the transgenic animals. This may be due to integration of foreign DNA occurring after the single-cell stage, which would result in animals with integrated foreign DNA in some cells but not in others (mosaic animals). Consequently, this technique is less commonly used for transgenic animal production.

In 1980, Gordon and colleagues published the first report of a transgenic mouse strain produced from microinjection of DNA into the pronucleus of a fertilized single-cell embryo. Although the precise mechanism by which foreign DNA is integrated into the genome by this technique is not known, the microinjection technique is frequently used in transgenic animal production because of its relative efficiency in generating transgenic animal lines that consistently express the integrated foreign gene. This technique is not without disadvantages however. For example, a percentage of microinjected embryos are not viable for re-implantation into foster mice, injected DNA does not always integrate into the host genome, and altered integration can occur which results in rearrangements or deletions of required host DNA sequences. As a result, it is necessary to work with large numbers of embryos in order to generate a transgenic animal line via microinjection. Other methods used in the production of transgenic animals include the insertion of foreign DNA into embryonic stem (ES) cells (Knapp and Kopchick 1994) and the use of spermatozoa as a vector for foreign DNA (Lavitrano et al., 1989). ES cell technology for the production of deletion mutations in mice is common, whereas the use of spermatozoa remains controversial (Knapp and Kopchick, 1994).

Refinements of the microinjection technique have made it a common and readily accepted technique in the production of transgenic animals (Boyd and Samid, 1993; Brinster et al., 1982). This is best evidenced by the abundance of literature reporting successful establishment of transgenic animal lines, via microinjection, for a variety of purposes. In just over a decade since its conception, transgenic technology has been employed to aid in our understanding of the biology of normal and pathological states. For example, several transgenic models have been used to examine aspects of gene regulation such as hormone and metal response elements located in gene promoter/regulatory regions (Palmiter et al., 1982a, 1982b, 1993, Valera et al., 1993). This has enhanced our understanding of the role nutrients play in modulating gene expression and has stimulated investigation to further clarify nutrient/gene interactions. Numerous transgenic models have been developed to investigate the proliferative action of oncogenes (Connelly et al., 1989), the efficacy of gene replacement therapy (Hammer et al., 1984), the consequences of overexpressing proteins such as cell receptors (Hoffman et al., 1988) and hormones (Valera et al., 1993; Knapp and Kopchick 1994) and for the production of recombinant proteins for pharmaceutical use (Pursel et al., 1989).

In order to assign a biological function to CRIP, additional information about its metabolism is required. To provide a model for further study of the potential functions of CRIP, transgenic mice were created which enable investigation of the effects of CRIP overproduction. This is significant because there is currently no known mechanism for increasing CRIP expression in a whole animal system.

Zinc Biology

The biological and nutritional significance of zinc centers around the vast number of metabolic processes this trace element is involved in through its interactions with hundreds of proteins in microorganisms, plants and animals. The average adult human body contains 1.5-2.5 g of zinc. Very sophisticated mechanisms exist to maintain zinc homeostasis, including absorption, transport, utilization and excretion. Presumably, this enables the appropriate distribution of zinc for efficient execution of its diverse biologic functions.

The mechanisms responsible for zinc absorption have been the subject of considerable investigation, and several review articles have been published summarizing the large body of literature generated by these studies (Cousins 1982; 1985; 1989). Zinc is absorbed from the small intestine, and it has been shown in humans, that the highest rate of zinc absorption occurs in the jejunum (Lee et al., 1989). Despite intensive study in this area, the mechanism of zinc absorption has not been elucidated. Both saturable, carrier-mediated and nonsaturable, nonmediated components are thought to be involved (Cousins, 1996). Transcellular zinc movement across the epithelial cell puts zinc in contact with several potential binding proteins, including CRIP. Whereas the involvement of CRIP in zinc absorption is unlikely, as described above, the association of zinc with metallothionein (MT) in the intestine is well documented (Cousins, 1985). Dietary zinc levels directly influence MT expression and zinc absorption is negatively correlated with intestinal MT levels suggesting that this protein is involved in regulating intestinal zinc absorption (Hoadley et al., 1988). Efflux of intestinal zinc into the lumen is also involved in zinc homeostasis (Hoadley et al., 1987). Portal circulation of newly absorbed zinc occurs via albumin (Smith et al., 1979). Distribution of zinc throughout the body occurs again via transport by albumin, although α-macroglobulin and several other proteins carry measurable amounts of zinc as well (Cousins and Hempe, 1990; Cousins, 1996).

Physiologic Functions

The essentiality of zinc in human nutrition has been recognized for the past decade (Raulin, 1869). However, biological roles for this trace element have been established only within the past 50 years. Zinc functions in biology in catalytic, structural and regulatory roles (Cousins, 1996). Underscoring the significant catalytic role zinc plays, is the fact that there are currently several hundred microbial, plant and animal metalloenzymes known to require zinc for optimal activity (Vallee and Falchuk, 1993). These enzymes are members of all six enzyme classes and are involved in the metabolism of proteins, carbohydrates, lipids and nucleoproteins, demonstrating the breadth of this trace element's involvement in biologic processes. Specific examples of enzymes requiring zinc include carbonic anhydrase, alcohol dehydrogenase and RNA polymerases.

The prevalence of zinc as a structural component of proteins further illustrates its physiologic importance. More than ten classes of zinc-containing structural motifs, present in thousands of proteins, have been identified and at least partially characterized (Klug and Rhodes, 1987; Schwabe and Klug, 1994). Zinc-binding enables the folding of relatively short stretches of polypeptide into defined units that would otherwise be energetically unfavorable. Two unique properties of zinc, which enable it to play key structural roles in such a large number of proteins with vastly differing functions, are its lack of redox activity and ability for rapid ligand exchange.

Zinc finger domains, where cysteine and histidine side chains coordinate zinc ions, are present in up to 1% of all human gene products (Berg and Shi, 1996). The zinc fingers of many of these proteins characteristically bind specific DNA target sites and act as transcription factors, for example retinoic acid and calcitriol receptors (Cousins, 1996).

Because of their ability to target specific DNA sequences, zinc fingers are currently being evaluated for use in therapeutic applications (Wu et al., 1995). Some zinc finger proteins interact with RNA and may bind to molecules other than nucleic acids as well (e.g. proteins). For example, the zinc fingers of the LIM protein, human cysteine-rich protein (hCRP) can homodimerize demonstrating that the double zinc finger domain can be involved in protein-protein interaction (Feuerstein et al., 1994).

Zinc also acts as a regulator of transcription. Through interactions with a metal-binding transcription factor, zinc is able to up-regulate metallothionein (MT) transcription (Cousins, 1994, Palmiter, 1994). A metal response element present on mammalian MT promoters confers dietary zinc sensitivity to this gene. This characteristic has been exploited for use in transgene constructs by linking the MT promoter to a gene of interest. Expression of the transgene can then be regulated by dietary zinc intake or via zinc or cadmium injections. Recently, two acute phase proteins, C-reactive protein and α_1-acid glycoprotein, have been shown to be regulated by metals, presumably through metal response elements (Yiangou et al., 1991). The existence of other genes which may be regulated by zinc is currently being investigated through the use of differential display analysis of intestinal mRNA in response to dietary zinc treatments (Blanchard and Cousins, 1996).

Clearly, because of their abundance, metalloenzymes and zinc finger proteins contribute significantly to the dietary zinc requirement. The extent to which dietary deficiency or excess influences the activity of these proteins needs to be established. Further investigation of this relationship could reveal useful markers for assessment of zinc nutriture as well as help to identify mechanisms responsible for deficiency and toxicity symptoms

Zinc Requirements and Status Assessment

The recommended dietary allowance (RDA) for zinc is based on an assumed fractional absorption of 20% (National Research Council, 1989). Data from balance studies and estimations of zinc losses in healthy adults suggest that an average zinc absorption of 2.5mg/d is required in order to maintain equilibrium. This translates to an RDA for zinc of 15 mg/day for adolescent and adult males, 12 mg/d for adolescent and adult women, 10 mg/d for children under 10 years and 5 mg/d for infants. During pregnancy, when cellular proliferation and differentiation is increased, the RDA for women is increased to 15 mg/d. Based on the average content of human milk, a recommended allowance (adjusting for an absorption efficiency of 20%) of 19 mg/d and 16 mg/d has been set for the first and second six months of lactation.

Plasma zinc levels, measured by atomic absorption spectrophotometry, are often used for assessment of zinc status in humans and animals. However, changes in plasma zinc are representative of the size of the exchangeable pool of zinc and do not accurately reflect fluctuations in whole-body zinc (King, 1990). This is because plasma zinc concentration is subject to homeostatic control and is consequently maintained at 0.8-1.2 µg/mL despite inadequate zinc intake. For example, in mild zinc deficiency, growth reduction and decreased zinc excretion result in the maintenance of normal plasma zinc concentrations despite negative zinc balance. An additional problem associated with the use of plasma zinc concentration as an index of zinc status is that it is reduced in response to stress, infection, meals, and some hormonal states. Short-term fasting can increase plasma zinc levels. Therefore, unless these variables can be eliminated, plasma zinc concentration as an indicator of zinc status is of limited reliability and sensitivity in mild zinc deficiency (Cousins, 1989, 1996). In severe zinc deficiency homeostatic control mechanisms are exceeded and altered transfer of zinc from bone, liver and other tissue allows the plasma zinc concentration to decline. The development of metabolic and clinical signs of zinc deficiency (as discussed below) would then support the diagnosis of zinc deficiency in conjunction with low plasma levels.

Synthesis of MT is regulated by dietary zinc intake, as described previously. Therefore, use of this protein as an index of zinc status has been investigated. Plasma and erythrocyte MT has been measured by radioimmunoassay (Sato et al., 1984) and, more recently, by enzyme-linked immunosorbant assay (ELISA) (Grider et al., 1989). Currently, the use of reverse-transcription-polymerase chain reaction (RT-PCR) is also being evaluated for the measurement of monocyte MT mRNA as an indicator of zinc status (Sullivan et al, 1996).

Deficiency and Toxicity

In humans, zinc deficiency is characterized by growth retardation, delayed sexual development, hypogonadism, dermatological lesions, anemia, hepatosplenomegaly and lethargy (Prasad et al., 1963). Delayed wound healing, impaired immune function, anorexia, alopecia, reduced taste acuity, emotional disorders and diarrhea are also associated with zinc deficiency (Aggett, 1989; Hambidge, 1989). All of the precise biochemical mechanisms responsible for the symptoms associated with zinc deficiency have not been defined. Further investigation of zinc-dependent proteins may help to elucidate these mechanisms.

Because of the mechanisms which efficiently maintain zinc homeostasis, mammals are capable of tolerating relatively high zinc intakes. However, acute ingestion of large quantities of zinc (>150 mg/d) may result in nausea, vomiting and dizziness (Fosmire, 1990). Chronic ingestion of 100-300 mg/d of zinc may potentiate copper deficiency, impaired immune response and altered plasma lipoprotein concentrations (Cousins, 1996).

Although a substantial amount of research has provided valuable insight into the critical role of zinc in physiological processes, much remains to be learned about the mechanisms through which zinc exerts its biologic effects. Through investigations of proteins such as CRIP, which most likely require zinc for their biologic activity, it is hoped that a more complete understanding of the role of zinc in metabolism will be realized.

CHAPTER 2
MATERIALS AND METHODS

Genomic Organization of Rat Cysteine-Rich Intestinal Protein

Isolation and Subcloning of CRIP Structural Gene

The CRIP structural gene was identified, isolated and subcloned from a clone previously screened from a rat intestinal genomic library in the Lambda Dash II vector (Levenson et al., 1994). Identification of the gene was accomplished by restriction mapping and Southern analysis of a clone thought to contain the entire CRIP gene and promoter/regulatory region. Typically, 1-5 μg of DNA were digested with 5-25 units of the appropriate restriction enzyme(s) purchased from either Promega (Madison, WI) or Gibco BRL (Gaithersburg, MD) using restriction enzyme 10X buffer supplied by the manufacturer. Reactions were incubated at 37°C for 2 h to overnight. Digestion mixtures were electrophoresed on a 0.75-1.5% agarose gel (UltraPure, Gibco BRL) to separate the DNA fragments by size. Following electrophoresis, the gel was stained with ethidium bromide to allow visualization of the DNA fragments generated by restriction digestions. The DNA was then transferred to a nylon membrane (GeneScreen, NEN Life Science Products, Boston, MA) by a modification of the Southern Blot procedure (Molenaar and Wilkins, 1991). First, the gel was equilibrated by gentle rocking in 0.25 N HCl for 10 min with one change of acid solution; then it was made alkaline by rocking in 2 changes of 0.4 M NaOH for 15 min each. Next, the gel was placed DNA side up and was covered with a piece of GeneScreen membrane that had been soaked in 0.4 M NaOH. The membrane was covered with filter paper and 6 cm of paper towel. A piece of glass was placed over the paper towel to produce a light, even pressure and the DNA was allowed to transfer by capillary action onto the GeneScreen overnight. Following the transfer, DNA was crosslinked to the membrane by UV irradiation.

Membranes were equilibrated for 2 h in hybridization solution (5X Denhardt's solution, 100 µg/mL salmon sperm DNA, 0.5% sodium dodecyl sulfate (SDS) and 6X SSC (10X SSC is 3M NaCl and 0.3 M $Na_3C_6H_5O_7{\bullet}2H_2O$) were incubated with probes overnight at 37-65° C. For cDNA probes, hybridizations were carried out at 65° C. However, for end-labeled oligonucleotide probes, hybridization and wash temperatures were calculated based on the number of A-T and G-C base pairs in the oligonucleotide. The optimum hybridization temperature (T_H) was determined by the formula $T_H = 2°$ C (A-T bp) + 4° C (G-C bp) - 5° C (manual accompanying GeneScreen). In either case, hybridizations were always carried out overnight. To remove any nonspecifically bound probe from the membrane, the following washes were performed: 5 min in 2X SSC and 0.5% SDS at room temperature, 15 min in 2X SSC and 0.1% SDS at room temperature with gentle agitation, twice for 30-60 min in 0.1X SSC and 0.5% SDS at 37° C with gentle agitation and briefly in 0.1X SSC at room temperature. The membrane was then autoradiographed.

Preparation of Radiolabeled Probes

cDNA probes were labeled using the Random Primers DNA labeling system (Gibco BRL) according the the protocol supplied by the manufacturer. Briefly, 25 ng of double-stranded CRIP cDNA, dissolved in 10 µL of distilled H_20, was denatured by boiling for 5 min then immediately placed on ice. A reaction mixture was prepared containing the denatured DNA plus 6 µL of a dNTP solution containing 0.5 mM each of dATP, dTTP and dGTP in 3 mM Tris-HCl, pH7.0 and 0.2 mM Na_2EDTA, 15 µL

Random Primers Buffer Mixture (0.67 M HEPES, 0.17 M Tris-HCl, 17 mM $MgCl_2$, 33 mM 2-mercaptoethanol, 1.33 mg/mL BSA) and approximately 50 µCi of [α-^{32}P] dCTP (NEN Life Science Products). Three units of Klenow fragment were then added and the mixture was incubated at room temperature for periods of 3 h to overnight. Unincorporated nucleotides were removed from the mixture using a Sephadex G-50 spin column (LKB Pharmacia, Piscataway, NJ) equilibrated with TE buffer (10 mM Tris-Cl, 1mM EDTA, pH 7.4). A 1 µL aliquot was then used for determiniation of total radioactivity by liquid scintillation counting. The probe was then boiled for 5 min and added directly to hybridization solution.

Oligonucleotide probes were end-labeled with [γ-^{32}P] -ATP (NEN Life Science Products) by incubating 120 ng of oligonucleotide with 10 units of T_4 polynucleotide kinase and 10X kinase buffer (0.5 M Tris-Cl, pH 7.6, 100 mM $MgCl_2$, 50 mM dithiothreitol, 1 mM EDTA) for 30 min at 37° C. The kinase was inactivated by heating at 65° C for 5 min and unicorporated radionucleotide was removed by passing the mixture through a Sephadex G-50 column as described above.

Subcloning of CRIP Structural Gene

The DNA fragment containing the stuctural gene was digested with the restriction enzymes EcoRI and XhoI, separated by electrophoresis and the DNA fragment determined to contain the entire CRIP structural gene was excised from a 1% agarose gel and purified using either Wizard Prep (Promega, Madison, WI) or Qiaquick Gel Extraction Kit (Qiagen, Germany). The purified EcoRI/XhoI DNA fragment was ligated into the EcoRI and XhoI sites of the pGEM-11Zf(+) vector (Promega). This vector was prepared for ligation by digestion with the restriction enzymes EcoRI and XhoI. One hundred nanograms of digested vector was incubated with 300 ng of the digested and purified DNA fragment, 10X ligation buffer and T_4 DNA ligase (Gibco BRL). The ligation reaction was incubated at 14°C overnight.

Transformation of competent cells. Immediately following overnight incubation, ligation mixtures were transformed into Subcloning Efficiency DH5α™ Competent Cells (Gibco BRL) according to the protocol suggested by the manufacturer. Three microliters of ligation mixture was added to 50 μL of competent cells in chilled, sterile 1.5 mL microcentrifuge tubes and mixed gently. The cells were incubated for 30 min on ice, heat shocked for 20 s at 37° C then returned to ice for 2 min. Following this, 950 μL of Luria broth (LB) at room temperature was added and the cells were incubated with shaking (225 rpm) for 1 h at 37° C to allow for expression from the transformed plasmid. The cells were then vortexed and 100 μL of the transformation mixture was spread onto 100 mm LB-agar plates containing 100 μg/mL ampicillin and incubated at 37°C overnight. In some instances the plates contained 1mM X-gal and 200 μg/mL IPTG to allow for blue-white screening of the transformants. Colonies were grown in 3 mL LB in a shaking water bath at 37° C overnight.

Plasmid preparation. Plasmids were purified from overnight cultures using the Mini-Boiling method (Holmes and Quigley, 1981). Cultures were centrifuged in 1.5 mL aliquots in a microcentrifuge to pellet cells. Supernatant was removed and the pellet was resuspended by vortexing in 550 μL STET buffer (8% sucrose, 5% Trition X-100, 50 mM EDTA, 50 mM Tris-HCl, pH 8.0) and 25 μL lysozyme solution (10mg/mL in sterile H_2O). Following vortexing, the tubes were placed in a boiling H_2O bath for 40 s then immediately centrifuged for 10 min. The gelatinous pellet was removed and 600 μL of isopropanol (-20° C) were added to the supernatant. After inverting tubes to mix, they were centrifuged for 10 min. The resulting pellet was dissolved in 100 μL TE (10 mM Tris-HCl, pH 8.0, 50 mM EDTA); then 7 μL of 5 M NaCl were added followed by 250 μ

L cold 95% ethanol to precipitate the DNA. After mixing well, the samples were microcentrifuged for 10 min at 4° C. The supernatant was removed and the last traces of ethanol were carefully removed with a gel-loading pipet tip. DNA pellets were allowed to dry for no more than 5 min and then were resuspended in TE, pH 8 or sterile ddH_2O. The identity of the plasmids was verified by digestion with restriction endonucleases followed by size separation of the DNA via gel electrophoresis. The plasmid consisting of pGEM11-Zf(+) DNA and the 5kb CRIP-containing DNA fragment were named pCRIP5.

Nested Deletions for Sequencing

Regions longer than 500 bp are too long to be sequenced using a single primer and the CRIP coding region was estimated to be approximately 2 kb in length. For this reason, the pCRIP5 plasmid was subjected to Exonuclease III (ExoIII) digestion using the Erase-a-Base System (Promega). This enabled unidirectional deletions to be made from the 5' end of the CRIP genomic sequence, allowing for the production of smaller constructs which could be sequenced (see figure 2-1). First, the pCRIP5 plasmid was digested with the restriction endonuclease SfiI (Gibco BRL) which linearized the plasmid and resulted in a 3' recessed end. To protect this end from ExoIII digestion, it was filled in with alpha-Phosphorothiolate nucleotides using the Klenow fragment of DNA Polymerase I (Promega). The plasmid DNA was purified from these reactions by phenol:chloroform extraction followed by ethanol precipitation and resuspension in TE buffer (10 mM Tris, 0.5 mM EDTA, pH 7.4). Next, the plasmid was digested with the restriction endonuclease EcoRI to create a suitable end for ExoIII digestion. These digestions created a linearized plasmid that was resistant to digestion by ExoIII on the 3' end and susceptible to digestion by this enzyme on the 5' end of CRIP. Since ExoIII has a processivity of approximately 450 bp/m at 37°C, incubating the digested DNA fragment

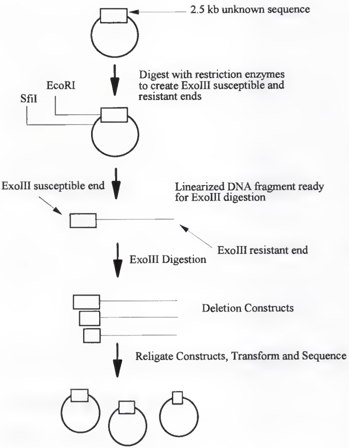
2.5 kb unknown sequence
Digest with restriction enzymes to create ExoIII susceptible and resistant ends
EcoRI
SfiI
ExoIII susceptible end
Linearized DNA fragment ready for ExoIII digestion
ExoIII resistant end
ExoIII Digestion
Deletion Constructs
Religate Constructs, Transform and Sequence

Figure 2-1: Scheme for construction of deletion contructs.

with ExoIII for varying lengths of time enabled the production of DNA deletion constructs of varying lengths. In this manner, sequential segments of the CRIP coding region were deleted and as a result, the sequencing priming site was moved further into the coding region. Following the deletions, plasmids were religated to produce circularized deletion-containing vectors. These vectors were used to transform DH5α cells (Gibco BRL) as described above. Plasmids were then prepared from each deletion construct and then sequenced.

Sequencing Reactions

The chain-termination sequencing method with the Sequenase Version 2.0 DNA Sequencing Kit (USB, Cleveland, Ohio) was used to determine the sequence of the CRIP structural gene. This method relies on the *in vitro* synthesis of DNA using an oligonucleotide primer which initiates synthesis at only one site on a DNA template under the direction of Sequenase v.2.0, T7 DNA Polymerase. This method follows a two-step procedure, whereby a primer is extended using a limiting concentration of deoxynucleoside triphosphates (dGTP, dCTP and dTTP) and radioactively labeled [^{35}S] dATP (NEN Life Science Products) in the first step. The second step involves the incorporation of dideoxynucleotides into the synthesizing chain. Incorporation of these nucleotide analogues results in termination of synthesis because they lack the 3'OH group which is essential for chain elongation.

Ten microliters of plasmid DNA was denatured in 4μL 2M NaOH, 2mM EDTA and 26 μL ddH$_2$O at 37°C for 30 min. The reaction was neutralized by adding 4 μL of 3M Sodium Acetate and the DNA precipitated by adding 88μL 95% ethanol and incubating at -70°C for 15 min. The DNA was pelleted by centrifugation for 10 min and the pellet was washed with 70% ethanol then resuspended in 14 μL ddH_2O. Seven microliters of denatured template was used per sequencing reaction.

Two microliters of reaction buffer (5X is 200 mM Trsi-HCl, pH 7.5, 100 mM $MgCl_2$ and 250 mM NaCl) and 1 μL of Primer (M13 forward or reverse) were added to 7 μL denatured template in a 500 μL microfuge tube and annealing of primer to template was carried out for 2 min at 65°C. The reaction was allowed to slowly cool over a period of 15-30 min by placing the tube in a beaker of 65°C water and allowing it to cool to room temperature. Once annealing was completed (after temperature was below 35° C), the tube was placed on ice and used within 4 h.

To the 10 μL of ice-cold annealed DNA mixture, the following was added: 1.0 μL 0.1 M dithiothreitol (DTT), 2.0 μL diluted labeling mix (2 μL of labeling mix [5X concentrated is 7.5 μM dGTP, 7.5 μM dCTP, 7.5 μM dTTP] was diluted with 8 μL H_2O), 0.5 μL $[^{35}S]$ dATP, and 2.0 μL diluted Sequenase enzyme (1.0 μL Sequenase, 0.5 μL pyrophosphatase and 6.5 μL enzyme dilution buffer [20 mM Tris-HCl, pH 7.5, 2 mM DTT, 0.1 mM EDTA and 50% glycerol]). This labeling reaction was allowed to incubate at room temperature for 2-5 min.

To terminate the labeling reaction, 3.5 μL of labeling reaction was transferred to each of 4 tubes which had been labelled (G, A, T, C), filled with 2.5 μL termination mixture (80 μM dGTP, 80 μM dATP, 80 μM dCTP, 80 μM dTTP, 50 mM NaCl and 8 μM of either ddGTP, ddATP, ddTTP or ddCTP) and pre-warmed at 37°C. The termination reactions were mixed well and incubated at 37°C for 5 min. To this reaction, 1 μL of TdT cocktail (2.0 μL 5X TdT buffer, 2.5 μL ddH_2O and 0.5 μL TdT) was added and this mixture was then allowed to incubate for 30 min at 37°C. To stop the reactions, 4 μL stop solution (95% formamide, 20 mM EDTA, 0.05% Bromophenol Blue and 0.05% Xylene Cyanol FF) were added.

A 6% denaturing acrylamide gel was prepared and a 38 x 50 cm Sequi-Gen Nucleic Acid Sequencing Cell (Bio-Rad Laboratories, Richmond, CA) was used to electrophoretically separate sequencing reactions. Samples were heated at 75°C for 2 min immediately prior to loading onto the sequencing gel. Electrophoresis was carried out at 75 watts for 8 h. The gel was then dried with a vacuum and exposed to film (X-Omat, Kodak) for 3 days.

The services of the DNA Sequencing Core Facility at the University of Florida were also utilized for determination of sequence information using Taq DyeDeoxy Terminator Cycle Sequencing.

Production of CRIP Transgene (CRIPTG) Construct and Transgenic Mice

Polymerase Chain Reaction

The Poylmerase Chain Reaction (PCR) was used to amplify ~ 1.75 kb of the CRIP promoter/regulatory region and ~ 2.5 kb of the CRIP coding region from pCRIP5 plus 91 bp of vector sequence to produce a DNA construct (CRIPTG) for microinjection to produce transgenic mice. The vector sequence was included in the construct to enable identification of transgenic mice. This was necessary because the rat and mouse CRIP genes are highly homologous. The upstream 5' CCA GAG GTC CTG AGT TCA AT and downstream 5' CAG CTA TGA CCA TGA TTA CGC C 3' PCR primers were designed using the GCG OSP primer analysis program (Program Manual - Genetics Computer Group, 1994) based on data from genomic sequencing of the CRIP gene that has been described above. Primers were synthesized by the University of Florida ICBR DNA Synthesis Core and by Gibco BRL. The PCR reactions were were carried out in 20 μL volumes using the *Thermus aquaticus* (*Ampli*Taq) DNA polymerase. Reactions included

1 ng of pCRIP5 plasmid DNA as the template, 2.0 μM upstream primer, 2.0 μM downstream primer, 2.5 mM dNTPs2.5 U *Ampli*Taq DNA polymerase (Perkin Elmer, Branchburg, NJ) and sterile water. After an initial 2 min denaturation at 94° C, samples were subjected to 30 cycles of 94°C for 30 s, 60°C for 1 min and 72°C for 2.5 min, followed by a final extension at 72°C for 4.5 min. An aliquot from the PCR reaction was then electrophoresed on a 1% agarose gel for analysis of the products. This reaction yielded ~ 5kb product as expected.

Cloning of PCR Product

To produce large quantities of the CRIPTG construct, the ~ 5kb PCR product was ligated into the pCRII vector using the TA Cloning kit (Invitrogen, San Diego, CA). PCR products generated using *Ampli*Taq DNA polymerase have a single 3' A-overhang. The TA Cloning vector exploits this terminal transferase activity by providing a single 3' T-overhang which enables direct ligation of PCR products. To clone the CRIPTG PCR product, 3 μL of the PCR product was added to the ligase, ligase buffer, pCRII vector and water to a final volume of 10 μL and was incubated at 14°C overnight. Transformation reactions and plasmid isolations were carried out as described earlier in this chapter. For transfections, the pCRIPTG plasmid was used without further purification. However, for microinjection into mouse embryos, additional purification procedures were followed as outlined below.

Purification of CRIPTG Fragment

Successful transgene integration depends upon the purity of the injected DNA . Therefore, it is imparative that any impurities be completely removed from the fragment. In order to obtain a purified transgene construct the following procedure was used. First, the CRIPTG construct was excised from the pCRII vector using the enzyme *EcoRI* (Gibco BRL). Next, to separate this insert from the vector, the restricition digests were electrophoresed on a 1% agarose gel at 40V for 4 h. Approximately 1.5 μg of DNA was loaded into each well. It was necessary to run the gel at low voltage for this length of time because the CRIPTG construct and pCRII vector were of similar lengths. These electrophoresis conditions gave complete separation of insert and vector, which prevented contamination of the transgene construct with vector. Following electrophoresis, the gel was stained in 1 ng/mL ethidium bromide to allow visualization of the DNA bands under UV light. Care was taken to limit exposure of the DNA to UV light to prevent degredation of the transgene construct. Using a sterile razor blade, the ~5 kb transgene band was quickly excised from the agarose gel and transferred to a pre-weighed, 2 mL microcentrifuge tube.

The DNA was immediately purified from the agarose slice using QIAquick Gel Extraction columns (Qiagen) according to the protocol suggested by the manufacturer. First, the weight of the gel slice was determined so that less than 400 mg gel were used per column. Next 3 volumes of solubilization buffer QX1 (Qiagen) was added to 1 volume of gel (100 mg ~ 100 μL) and the mixture was incubated at 50° C for 10 min. To help dissolve the gel, the tube was inverted and tapped every 2-3 min during the incubation. Once the gel was solubilized completely, 1 gel volume of isopropanol was added. This mixture was then loaded on a QIAquick spin column that had been placed in a 2 ml collection tube. The column was centrifuged at 10,000 x g for 1 min in a table-top microcentrifuge tube. To ensure complete removal the agarose, an additional 0.5 mL of QX1 buffer was spun through the column. The column was then washed with 0.75 mL of wash buffer PE (Qiagen) for 1 min at 10,000 x g followed by a 1 min spin to ensure removal of the PE buffer. The spin column was transferred to a 1.5 mL collection tube and 30-50 μL of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) was applied to the column, allowed to stand for 1 min then centrifuged for 1 min as above. Approximately 500 ng of purified CRIPTG construct was recovered from each spin colum purification. Eluate from 4 spin columns was then pooled and re-purified by running through a QIAquick column again, or by purification through an Elutip-d minicolumn (Schleicher & Schuell, Keene, NH) according to the manufacturer's suggested protocol. After purification, the concentration of the CRIPTG sample was determined by spectrophotometry at 260 nm and confirmed by running 1 and 5 μL aliquots on a 1% agarose gel alongside molecular mass markers (Gibco BRL).

Verification of pCRIPTG Identity and CRIP Expression from Construct

Following the cloning of the PCR product, its identity was confirmed in four ways. First, the pCRIPTG, at a concentration of 1 ng/20 μL reaction, was used as a template in a PCR reaction under the conditions used for production of the construct. This reaction resulted in a product which migrated on a 1% agarose gel identically to the original CRIPTG product. Next, restriction mapping of pCRIPTG was carried out using restriction endonucleases known to cut the transgene construct. The enzymes EcoRI, BglII, XbaI and XhoI (Gibco BRL) produced appropriately sized bands when digestion reactions of pCRIPTG were electrophoretically separated on a 1% agarose gel next to 1 kb DNA size markers. Third, pCRIPTG was digested with the restriction endonuclease EcoRI to liberate CRIPTG from the pCRII plasmid. Following electrophoresis of this digestion reaction, a Southern transfer of the DNA was performed and the blot was probed with a CRIP-specific oligonucleotide. The probe hybridized to the transgene band but not to the plasmid. Finally, a portion of the pCRIPTG plasmid was sequenced.

To confirm the ability for CRIP mRNA expression from the CRIPTG construct, cultured rat intestinal epithelial cells (IEC-6) were transfected with the pCRIPTG plasmid. IEC-6 cells were obtained from American Type Culture Collection (Rockville, MD) at passage 14. The cells were maintained in 100 mm culture dishes in complete medium - Dulbecco's modified Eagle's Medium (DMEM) supplemented with glutamine, insulin (0.1 u/mL), 10% FBS and an antibiotic/antimycotic preparation (Gibco BRL, Gaithersburg, MD)) at 37° C, 5% CO_2. The cells were passaged 1:4 with 2 mL 0.25% trypsin in Hank's buffered saline (Gibco BRL) per plate 1-2 times per week.

Cells were plated at a density of ~ 7.5×10^5 per 35 mm culture dish 18-24 h before transfection, this resulted in cells at approximately 50-75% confluency on the day of transfection (Levenson et al., 1994b). Immediately prior to transfection, cells were washed 3X with serum-free medium (complete medium minus FBS), 0.5 mL serum-free medium was then added to each plate of cells. Three micrograms of plasmid DNA was transfected per 35 mm plate of cells using 2 μL Transfectam (Promega) reagent per microgram DNA by mixing both with serum-free medium prior to gently overlaying the transfection mixture onto cells. The transfection mixture was incubated with the cells for 5-12 h, then 4.5 mL complete medium were added. The cells were transfected with the pCRIPTG plasmid or with a pβ-Gal plasmid (Promega) to monitor transfection efficiency. Generally, transfected cells were harvested 24-48 h post-transfection for determination of CRIP mRNA levels (as described below) and β-Galactosidase activity.

β-Galactosidase activity was measured in cell extracts by monitoring the transformation of *O*-nitrophenol-β-D-galactosidase (ONPG) to galactosidase and *O*-nitrophenol (Promega). Briefly, cell extracts or β-Galactosidase standards were incubated for 3 h to overnight in assay buffer (200 mM sodium phosphate, pH 7.3, 2 mM $MgCl_2$, 100 mM β-mercaptoethanol, 1.33 mg/mL ONPG) at 37° C. Absorbance was then read at A_{415} after the addition of 1M Na_2CO_3.

Production of Transgenic Mice by Microinjection

Microinjection of the CRIPTG construct was performed by the National Institute of Child Health and Development (NICHD) Transgenic Mouse Development Facility at the University of Alabama at Birmingham. Briefly, this involved the microinjection of the CRIPTG DNA at a concentration of approximately 1-2 ng/µL into C57BL/6J x SJL/J F2 hybrid mouse zygotes (Polites and Pinkert, 1994). The surviving eggs were then reimplanted into pseudopregnant recipient mice. Three weeks after birth, these mice were weaned and tail biopsies were performed. DNA was extracted from the tail tissue and was used for PCR identification of founder transgenic mice.

Identification of CRIP Transgenic Founder Mice and Transgenic Offspring

Genomic DNA was obtained from the tails of potential founder mice according to a modification of the method of Gordon (1993). Approximately 1-2 cm of mouse tail was removed from 3 week old mice. At the time of tail biopsy, an earpunch was used to label each animal. This enabled identification of transgenic and control animals following analysis of genomic DNA for transgene integration. The tail was cut into 3-5 pieces and placed in a 2 mL microcentrifuge tube with 500 µL of tail biopsy buffer (10 mM NaCl, 10 mM Tris-HCl, pH .0, 10 mM EDTA, pH 8, and 2% SDS). Fifty microliters of proteinase K (20 mg/mL in water, Fisher Scientific, Pittsburgh, PA) was added and the mixture was incubated at 37° in a shaking water bath for 2-3 h. In the last h of incubation, 10 µL RNAse A (1 mg/mL, Fisher Scientific, Pittsburgh, PA) was added. Following incubation, 300 µL Tris-saturated phenol was added to each tube, mixed well by shaking and microcentrifuged for 15 min at 12,500 x g at 4° C. The viscous supernatant was then harvested with a 200 µL pipette tip that had been cut with a razor to produce a wide opening. This was necessary to prevent shearing of the high molecular weight DNA. The supernatant was then extracted with 150 µL phenol and centrifuged and harvested as above. This supernatant was extracted with 100 µL chloroform-isoamyl alcohol (24:1, v/v). DNA was precipitated from the aqueous phase with 2.5 vol of ice-cold 100% ethanol, washed 3 times with 70% ethanol and redissolved in 100-200 µL TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8). Typical yields of 100 µg genomic DNA, suitable for PCR or restriction digest analysis, was obtained by following this protocol.

The polymerase chain reaction (PCR) was utilized for identification of transgenic founder mice. Forward, 5' CAT GCC ACT GAC CAT TC 3' and reverse, 5' CCA TGA TTA CGC CAA GC 3' PCR primers were synthesized which allowed amplification of an 800 base pair product from the genomic DNA of transgenic mice but did not amplify a product from the non-transgenic mouse genome. This was accomplished by making the reverse primer complimentary to a section of the vector sequence which was present in the CRIP transgene but not the endogenous CRIP gene. Twenty microliter PCR reactions were performed as described above, using 0.5 µL of genomic tail DNA as a template to identify transgenic founder mice. All DNA samples were analyzed at least two times to confirm the presence or absence of transgene in the genome. Mice determined to be transgenic were mated to C57BL/6J x SJL/J F1 (The Jackson Laboratory, Bar Harbor, ME) to establish transgenic lines.

Animal Husbandry

Animals were housed at the University of Florida Transgenic Mouse facility under specific pathogen free (SPF) conditions and following guidelines established by the

Institutional Animal Care and Use Committee (IACUC). Microisolator cages (12x8x5 in.) were used to house no more than 5 mice per box. Water, bedding and cages were autoclaved and food was gamma-irradiated to prevent pathogen contamination. Cages were opened only under laminar flow hoods. These precautions were essential to prevent the spread of pathogens within the transgenic animal colony. Typically, mice were transferred to the Food Science and Human Nutrition Department animal room overnight prior to experimentation.

Analysis of Transgene Integration and Determination of Transgene Copy Number

For estimation of transgene copy number, 20 μg genomic tail DNA was spotted in duplicate onto a nitrocellulose membrane using a Schleicher & Schuell vacuum manifold. A standard curve of 0, 1, 5, 10 and 25 transgene copies/genome in genomic DNA were also applied in duplicate to the membrane. After UV crosslinking, the membrane was divided in two. One half was then probed with a rCRIP probe and the other half with a β-actin probe (to adjust for equal loading of DNA and hybridization efficiency). Densitometric determination of autoradiographs was made and hybridization intensity of the transgenic DNA (adjusted to actin intensity) was compared to the standard curve to estimate copy number. Densitometric analysis was accomplished by producing a digital photograph of the autoradiograph using a Kodak-CD40 digital camera. This image was then analyzed using Intelligent Quantifier v. 2.1 Electrophoresis Image Analysis Software (BioImage Systems Corporation, Ann Arbor, MI) which allowed for quantification of band intensities. Autoradiographs produced from Northern analyses of mRNA expression were quantified using this software as well.

Analysis of Transgene Expression

The ultimate purpose of establishing a CRIP transgenic mouse line was to provide a means of examining the effects of overexpression of CRIP. Therefore, the various lines of CRIP transgenic mice were evaluated for CRIP RNA expression to determine which line would be used for development of a colony of CRIP transgenic mice and used for establishment of transgenic animals homozygous for the transgene. Expression was examined in intestine, liver, kidney, heart, lung, brain, spleen, thymus, prostate, testes, ovary and uterus.

Northern analysis of CRIP mRNA

Total RNA was extracted from the tissues using TRIzol Reagent (GibcoBRL) according to the procedures outlined by the manufacturer. Immediately following harvesting, ~ 100 mg of tissue were homogenized in 3 mL TRIzol Reagent. Homogenized samples were kept on ice until all tissues were harvested. RNA was then either immediately extracted or samples were stored at -70°C for no more than 1 week prior to RNA extraction. The RNA pellet obtained from this extraction procedure was washed 2-3 times with 75% EtOH then dissolved in sterile diethylpyrocarbonate (DEPC) - treated water. Concentration and purity of the RNA samples were determined spectrophotometrically by measuring the absorbance at 230, 260 and 280 nm of 5 μL of sample diluted in 1000 μL TE.

Twenty micrograms of total RNA and 1 μl ethidium bromide were loaded per lane of a 1% agarose gel containing 0.66 M formaldehyde for Northern analysis. Following electrophoresis, RNA was capillary transferred and immobilized via ultraviolet cross-linking onto GeneScreen (NEN Life Science Products). Blots were pre-hybridized at 65° C for at least 1 h in 10-20 mL pre-hybridization solution (1% BSA fraction V, 1 mM EDTA, 0.5 M NaH_2PO_4, pH 7.4, 7% SDS) then probed with a CRIP cDNA probe prepared as described above. Equal loading of wells was confirmed by visualization of ethidium bromide staining of gels and by probing blots with a labeled 18S RNA or β-actin probe. Autoradiographs were densitometrically quantitated and comparisons among transgenic lines and between transgenic and non-transgenic animals were made.

Western, ELISA and immunohistochemical analyses of CRIP protein

Tissue homogenates for CRIP protein analysis by Western blot and ELISA were prepared by homogenizing tissues in 2-3 vol homogenization buffer (10 mM Tris, 154 mM NaCl, 10 μM $ZnSO_4$, 1 mM $MnCl_2$, 2 mM phenylmethanesulfonyl fluoride, 0.9 μg/mL pepstatin, 0.6 μg/mL leupeptin, pH 8.0) using a Potter-Elvejheim homogenizer immediately after removal from mice. Homogenates were centrifuged at 40,000 x g for 30 min at 4°C and the supernatant removed following this centrifugation was immediately stored at -70°C. Total protein was determined in supernatants by the Lowry method (Lowry et al., 1951). For Western analysis, equal amounts of total protein (40 μg/lane) were electrophoretically separated by SDS-PAGE (15% gel) and transferred to a nitrocellulose membrane (Nitro Bind, Micron Separations, Inc.). The membrane was placed in 5% non-fat dry milk in TBS-T (20 mM Tris, 154 mM saline, 0.05% Tween, pH 7.5) to block non-specific binding for 48 h at 4°C. The membrane was then incubated for 1 h at RT with a 1:100 dilution of a rabbit IgG raised against a rat CRIP peptide (amino acid residues 31-56) as used previously (Cousins and Clark, unpublished observations). Following this incubation, the membrane was washed 5 times in TBS.-T then incubated with secondary antibody, donkey anti-rabbit IgG linked to horseradish peroxidase (Sigma Chemical Co., St. Louis, MO) at a dilution of 1:10,000 for 1 h at RT. After repeated washing in TBS-T, the membrane was incubated with ECL reagent (ECL Western Blotting Detection Kit, Amersham Life Science, Arlington Heights, IL) for 1 m. Blots were immediately exposed to film for 15 s to 10 m. ECL, or enhanced chemiluminescence, is a non-radioactive method for detection of antigen which employs a specific primary antibody and a horseradish peroxidase-conjugated secondary antibody. The oxidation of luminol, catalyzed by a horseradish peroxidase/hydrogen peroxide reaction, causes an emission of light which is detected by autoradiography film.

A sandwich enzyme-linked immunosorbent assay (ELISA) developed for quantification of CRIP protein (Lorraine Lanningham-Foster, unpublished observations) was used to detect CRIP protein in tissues from transgenic and non-transgenic mice. Briefly, plates were coated with chicken anti-CRIP IgG, blocked with SuperBlock (Pierce, Rockford, IL) and sample was applied. Next, rabbit anti-CRIP IgG was applied followed by an anti-rabbit IgG conjugated to alkaline phosphatase. Addition of p-nitrophenyl phosphate allowed colorimetric detection of the antigen-antibody complex. Known amounts of rhCRIP were assayed on the same plate as samples to provide a standard curve for quantitation.

For histological examination and immunohistochemistry, tissues were fixed in 10% neutral buffered formalin and imbedded in paraffin. Five micron sections of the fixed tissues were then used for light microscopy and immunohistochemistry. Peritoneal macrophage were obtained by introducing 5 mL PBS into the peritoneal cavity of Metofane^{TM}-anesthetized mice using a 10 mL syringe fitted with a 16 gauge needle. The peritoneal cavity was massaged for 1-2 min and fluid was withdrawn via the same syringe. Following centrifugation at 200 x g for 10 min at 4°C, cells were resuspended in 1 mL complete DMEM and plated onto gelatin-coated coverslips for 24 hours. The cells were fixed with 2% paraformaldehyde, 0.1% Triton X-100. Tissue sections were deparaffinized and placed in absolute methanol with 0.3% hydrogen peroxide to inhibit endogenous peroxidase activity. Next, sections were treated with goat non-immune serum to block non-specific background before being incubated with anti-CRIP IgG. A rabbit antibody, raised against a synthetic peptide representing amino acids 31-56 of rat CRIP that had been coupled to keyhole limpet hemocyanin (Fernandes et al., 1996), was used with the Histostain-Sp^R kit (Zymed Laboratories, Inc., San Francisco, CA). This antibody has been used for similar staining of tissues in this lab (Fernandes et al., 1996 and Khoo et al., 1996) and has also been shown by Western immunoblot to be specific for CRIP (Fernandes et al., 1996). Sections were then incubated with an anti-rabbit, biotinylated secondary antibody which served as a linker between the CRIP antibody and a straptavidin-peroxidase conjugate. The addition of a substrate-chromagen solution (aminoethylcarbazole), which gives a red color, revealed the presence of peroxidase. Sections treated in this manner were then counterstained with hematoxylin. In addition, intestine, thymus, spleen and lymph node sections were stained with hematoxylin and eosin to verify retention of normal histology in tissues which demonstrated high levels of CRIP overexpression.

Flow Cytometry

Single-cell suspensions were prepared by removing thymus from anesthetized mice and immediately placing the tissue in 3 mL complete DMEM. The tissue was cut into several pieces then pushed through a stainless steel mesh tea strainer, using the plunger of a 5 mL syringe, into a 50 mL conical tube. Medium was used to rinse cells from tissue which remained in the mesh. Cells were centrifuged at 200 x g for 10 min at 4°C, the resulting cell pellet was washed with 10 mL medium and centrifuged again. Cells were resuspended in staining medium (1% BSA, 0.05% sodium azide in PBS) and counted using a hemacytometer.

Polypropylene tubes (12x74) were coated with FBS to prevent cells and antibody from sticking to the walls of the tube during the staining procedure. FITC-labeled rat anti-mouse CD4, PE-labeled rat anti-mouse CD8, FITC-labeled rat IgG and PE-labeled rat IgG antibodies were used (Pharmingen, San Diego, CA) to label thymus single-cell suspensions. For each reaction, $2x10^6$ cells were suspended in 50 μL staining medium, antibody was diluted in 50 μL staining medium (FITC-labeled antibodies diluted to 0.5 μ g/10^6 cells, PE-labeled antibodies diluted to 0.25 μg/10^6 cells) then added to the reaction and mixed gently but thoroughly with the tip of a pipette. Cells were incubated with antibody for 20 min in an ice-water bath in the dark. Following incubation, 1 mL staining medium was added to each reaction and this mixture was centrifuged at 200 x g for 5 min at 4°C. For double labeling, cells were resuspended in 50 μL staining medium, mixed with 50 μL of diluted antibody, incubated for 20 min and washed as above. After the second labeling, cells were washed a second time and resuspended in 500 μL staining medium. In all experiments, unstained, single-labeled and isotype-labeled cells were run as controls. When double-labeling of cells was performed, the first labeling was with the FITC-labeled antibody followed by the PE-labeled antibody to prevent stearic hindrance of the large PE molecule from interfering with labeling by the smaller FITC molecule. Flow cytometry analysis of labeled cells was performed at the ICBR Flow Cytometry Core Laboratory on the University of Florida campus using a Becton-Dickinson FACScan instrument.

Statistical Analysis

The unpaired t test was used to statistically compare CRIP mRNA expression between transgenic mice and non-transgenic littermates, as well as for comparison of lymphocyte populations between these two groups of mice. The InStat Instant Biostatistics software package was used for all statistical analyses (GraphPad Software, San Diego, CA).

CHAPTER 3
RESULTS

Genomic Organization of Rat Cysteine-Rich Intestinal Protein

Isolation and Subcloning of rat CRIP Structural Gene

A genomic clone, P1, previously shown to contain the CRIP promoter sequence (Levenson et al., 1994) was shown by Southern analysis, using a rCRIP cDNA probe, to contain the rat CRIP structural gene. Restriction mapping and subsequent Southern analyses of this clone identified a 5 kb EcoRI/XhoI restriction fragment which contained the entire rCRIP coding sequence. Figure 3-1 shows that probes specific to the 5' and 3' ends of the rCRIP cDNA hybridized to this 5 kb restriction fragment, indicating that the entire CRIP structural gene was contained within the fragment. The fragment was then cloned into the pGEM-11zf(+) vector and this clone, designated pCRIP5, was used for subsequent sequencing reactions.

Sequencing of rat CRIP Structural Gene

Sequence data from 11 fragments were analyzed using the GelAssemble program (GCG Sequence Analysis Software Package, Madison WI), which identified overlapping sequences and enabled the construction of a consensus rCRIP genomic sequence. Figure 3-2 shows the orientation of these overlapping fragments and figure 3-3 contains the actual sequence data. Comparison of the rCRIP cDNA sequence to the genomic

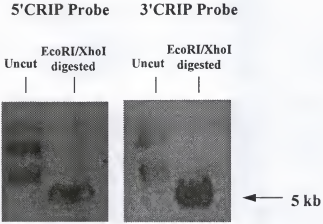

Figure 3-1: Southern analysis of P1 clone probed with oligonucleotide probes to the 5' and 3' ends of the rCRIP cDNA as described in Materials and Methods. This showed that the CRIP coding region was contained within the same clone as the rCRIP promoter.

```
Fragment Name                           Orientation

    800cf                                                     +---->
    D1jc48                                                  +----->
    430cf                                                +----->
    930bf                                            +------>
    D1jc54                                         <--------+
    D1jc47                                         <-----+
    D1jc58                                      +------->
    D1jc55                                  +------>
    28r                                +----->
    D1jc49                            +-------->
    D1jc52                        <--------+
    Consensus                     <----------------------------------+
                                 |----------|----------|----------|---------|
    Bases                        0          800        1600       2400  3200
```

Figure 3-2: Orientation of overlapping fragments of rCRIP genomic sequence. Fragments were sequenced then analyzed using the GelAssemble program to identify overlaps and generate a consensus sequence.

Figure 3-3: Genomic sequence of rat cysteine-rich intestinal protein coding region. Exons are noted by capital letters and are underlined. Various response element consensus recognition sequences are underlined and labelled.

```
      Transcription Start Site - Exon 1                                       Translation Start Site
      ↓                                                                       ↓
1     TCCAGAGCCT ACAACCTACT TCCTTCTAGC TGCAGCCACT TGTGCAGGAC CAGGTGCCGC CATGCCGAAG TGCCCCAAGT GCGACAAGGA
91    GGTGTATTTC Ggtgagcaca attgggtatc ccagcctctc tccacattgc ccgcgggcgt ttagcaggcg gtacgcggaa gcatggtcca
181   gaaacagaca atactgtgct tttcaggtgg accggagtgt ctagagtccg gggcatcgcc tgggttcgaa cccatggacc ttgcccgcgg
271   tttgagggtg ggataatttg gtgttaaacg gaaccgacat ctgtgcccac tcgctgtctt atgtttctgg gtcggtcttc tggcttcttt
361   gcctctgggt ttccaggttt ccgtgactcc cgtgcttctg gatctccccc tctctctgac cctggncttt ggatcctgct gtctctcccg
                        Sp-1
451   cgttctgtct gtgcgccctg ggcgggtcc ttgctggatg ggaaaggaac gcgggactgg ggtggcccct cccaatgcta agtagaaaca
541   gacgcccaaa accacgtccg gggggtacga tctcgggtgc tcgtttttct tgctgtttgc gcgcgctagg gctcctatga gaaagtacat
       Ap-1                                           Ap-2
631   gagtaagaga tccctttgtc cctgctgaga cgaagggtgc gcccaggttc agtgcagagg tcagaggact ttaaccggat ctacctagnc
721   ttagatcttc cagactcgtt ctcccccact cccaagtatg aaggcctctt cctctccagc cctacacgcc ctcgggggta atatccctga
811   ggtgatactt gaaaatgtcc caccaatgag ccgggttggg tggttgggtg gccgggtggc ccatgncttt agtcccagca ctttagtccc
                                                                            GRE
901   ggaaggcaga gaggcaagca gcatctatct ctgtgaktac aagatcagcc tggtctacag agtgagtkcc aggacaggca gaactgcaca
991   gaaaaatccc gtctcaaaaa aaaaaaaata caaaaaacaa aaacaaaaaa cctaagataa aatatcccaa caacaggcga tccttctgtc
1081  agaggcagtc ccgggaccca gggtgggctg tcacattgag agaataaagt gtaggtgtct gtgcagcggg agagggtatg cagggactcc
                                                  Exon 2
1171  ggtgtctcat agtggggtct gtacttctgc agCTGAGCGA GTGACGTCAC TAGcCAAGGA CTGGCATCGT CCCTGCCTGA AGTGCGAGAA
1261  ATGTGGAAAG ACACTGACCT CTGGGGGTCA TGCTGAGGTA AGCGGCCGCT AGGGGGCAGC GGGAGGAGCC ATAGGAGCTG GGATGGAGGT
                                 Exon 3
1351  AACCCCTCTT CTTTTCTCTG TAGCATGAAG GCAAGCCCTA CTGCAACCAT CCCTGCTACT CCGCCATGTT TGGGCCCAAA Ggtatgtcgt
1441  tggacctttt ccctaacggg cctcccagag ctggcctccc tccacaacat attggtatcc ttattttttt tttttaatc ttttcttttt
           Exon 4
1531  ttgcagGCTT TGGGCGAGGT GGAGCTGAAA GCCACACTTT CAAGTAGACT GAGgtatagc cccacctcca gtcccacaca gtctataaga
                                                                                    Exon 5
1621  cagagtcacc cctaacagag taagtaacga aatgttgcac tcatctttgt ctctgtttct tcttacagGT TGTGGAAACT CTCCCTACCC
                                                                         Polyadenylation Signal
1711  GCCCAGGCGA GTGCCAGGCC TTGTCCCTAG ACAGCAGGGC TCTCCGCAGC CCTCCATGCC TTTAATAAAC TTGATCTTTG Gaatgcctgt
1801  gtttttgtct actggtgtgt gatggagggg agctgcagtg ggagcctcaa ccccaggcct aatgcaggac actgcttttc ctgttgttgc
1891  aggcagtgtt gcccagcacc ctttgaaagg atggaggggc atgagttcag gcctacatcc aactctggsa tccatgaagt tcctatagtt
1981  tgattctgta gccctgtggt gaggccctga ctgtcagggt cagcaggaac atggcttagg tggggctggc cactgttcag agggtgaatc
2071  agtgggagtt ggcttggttc ccttttttgag ctgcttggtg gtgggggagt cgtgtggatt tcttgccttt tccattttca cttcctgcaa
2161  ccccaccctg tccccagaag tgcactcttg tgggtgagtt ctgcagctgc tcagcctgga cccagacact ttttccctga ccaggaatgc
2251  cctaggcaac ctggtgctct aatgggattt gagggaggca gttgaaaggg ctttctggaa actctcgtgg ggccaggcag ctctcagctt
2341  tgccttcccc atgccactga ccattcaaga tcctttggac ttaacaaggc cacaaaggtc tcagcagtgc tctggcctag yctacgttca
2431  yctttggcac ggacactgca ggaacaagcc tgacaggtta ttggtgagtc ccataagtgg tctgaagtgt agtgtgtgga aatncttgag
2521  gtggccgcaa tatggaggag aagaggccga ccttcggtat nagtttccta ctgggagggg tgtgggtatg tttagcgttc aaccagacc
```

consensus sequence enabled the identification of introns and exons within the gene. As shown in figure 3-4, the rCRIP structural gene contains 5 exons, or coding sequence, and 4 introns, or intragenic sequence. Further analysis of the genomic organization showed that the double zinc finger is not coded for within one exon. Rather, the nucleotides which code for amino acids comprising the LIM motif are contained in exons one, two and three. Located within intron 1 are several noteworthy sequences. A consensus recognition sequence for the transcription factor, Sp-1, is located from nucleotides 470-475 and a glucocorticoid response element (GRE) is located from nucleotides 455-465 as is noted in figure 3-3. Also located within intron 1 is a sequence with 78% identity to the mouse B1 repetitive element (data not shown).

Production of CRIP Transgene (CRIPTG) Construct and Transgenic Mice

A 5 kb CRIP transgene construct was produced by using PCR to amplify the CRIP promoter, structural gene and 91 bp of vector sequence from the pCRIP5 plasmid (see figure 3-4). The product was cloned into a vector and its identity and ability to express CRIP was established.

Verification of pCRIPTG Identity and CRIP Expression from Transgene Construct

Following the cloning of the PCR product, its identity was confirmed in four ways as described in Chapter 2. Each method verified the presence of CRIP sequence in the construct. Figure 3-5 shows that the 372 bases of data obtained by sequencing one end of the construct corresponded to the appropriate bases of the CRIPTG.

To determine whether the CRIPTG construct was capable of expressing CRIP mRNA, the pCRIPTG plasmid was transfected into a cultured rat intestinal epithelial cell line, IEC-6. This demonstrated that cells transfected with the pCRIPTG plasmid express

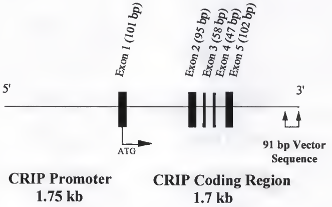

Figure 3-4: Map of CRIP transgene (CRIPTG) construct showing location of introns and exons. Vector sequence (91 bp) is located 3' to coding sequence to use for identification of transgenic mice.

more CRIP mRNA than cells transfected with a control plasmid, establishing the ability of this construct to express CRIP (figure 3-6).

Purification of CRIPTG for Microinjection

Once the identity of the CRIPTG construct was confirmed, it was excised from the pCRIPTG vector and purified. Electrophoresis followed by ethidium bromide staining and UV visualization was used to confirm the purity of the construct and to estimate its concentration (see figure 3-7).

Microinjection Statistics and Identification of CRIP Transgenic Founder Mice

The purified CRIPTG construct was microinjected into 187 C57BL/6J x SJL/J F2 hybrid mouse zygotes by the University of Alabama - Birmingham NTMDF. One-hundred fifty-four of these embryos survived microinjection and were implanted into 7 female mice. Forty-five mice were subsequently born, including 1 stillborn. DNA extracted from tails of these mice was sent to the University of Florida where it was analyzed in our lab by PCR to detect the presence of the transgene. A representative photograph of the PCR results is shown in figure 3-8. Six of the forty-five DNA samples were identified as containing the CRIP transgene. Five of those mice, two females (3611 and 3628) and three males (3617, 3626 and 3635), were used as founder mice and were mated to produce offspring which could be tested for transgene expression.

Development of CRIPTG Mouse Colony

Breeding founder mice. Each founder mouse was mated with a C57BL/6 x SJL/J F1 hybrid mouse to produce offspring. These breeding pairs are considered the F0 generation and their offspring are the F1 generation. Breeding was begun immediately following

```
pCRIPTG      73 CCAGAGGTCCTGAGTTCAATTCCCAGCAACCACATGGTGGCTC.ACAACC 121
                ||||||||||||||||||||||||||||||||||||||||||| ||||||
rCRIP Gene    1 CCAGAGGTCCTGAGTTCAATTCCCAGCAACCACATGGTGGCTCTACAACC 50
                         .         .         .         .         .
            122 ATCTGTGATGGGATCCGATGCCCTCTTCTGGTGTGCCCGAAGACAGCA.A 170
                |||||||||||||||||||||||||||||||||||||||||||||||| |
             51 ATCTGTGATGGGATCCGATGCCCTCTTCTGGTGTGCCCGAAGACAGCACA 100
                         .         .         .         .         .
            171 TGGTGTACTCACATACATAAAATAAATAAATAAATCTTAAAAAAGAAAGA 220
                ||||||||||||||||||||||||||||||||||||||||||||||||||
            101 TGGTGTACTCACATACATAAAATAAATAAATAAATCTTAAAAAAGAAAGA 150
                         .         .         .         .         .
            221 AAGAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAATGGCTGGGGGCCAGAG 270
                ||||||||||||||||||||||||||||||||||||||||||||||||||
            151 AAGAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAATGGCTGGGGGCCAGAG 200
                         .         .         .         .         .
            271 GGTGGGGTGGGGTGGGGCAGGCTGGTGGCCTTAGGTCCAGAAACCACTGG 320
                ||||||||||||||||||||||||||||||||||||||||||||||||||
            201 GGTGGGGTGGGGTGGGGCAGGCTGGTGGCCTTAGGTCCAGAAACCACTGG 250
                         .         .         .         .         .
            321 ACCTAAGGGGTGCCAACCCAGGCCTTTAGGTGGGTCTTAGGTAATGGTAG 370
                |||||||||||||||| |||||||||||||||||||||||||||||||||
            251 ACCTAAGGGGTGCCAAACCAGGCCTTTAGGTGGGTCTTAGGTAATGGTAG 300
                         .         .         .         .         .
            371 GCTAGAAGGTGGGAGTCTGTCCTTGTAGGTTCACCCCTGTTCTGGAGCTC 420
                ||||||||||||||:|||||||||||||||||||||||||||||||||||
            301 GCTAGAAGGTGGGANTCTGTCCTTGTAGGTTCACCCCTGTTCTGGAGCTC 350
                         .         .
            421 TCAAGGCTTCAGAAGGTAGAGC 442
                ||||||||||||||||||||||
            351 TCAAGGCTTCAGAAGGTAGAGC 372
```

Figure 3-5: Sequence comparison between pCRIPTG and 5' end of rat CRIP gene confirming the identity of the transgene construct.

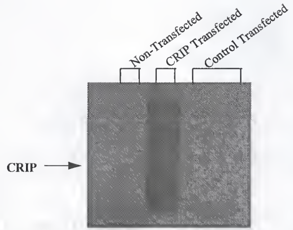

Figure 3-6: Northern analysis of mRNA from IEC-6 cells transfected with pCRIPTG plasmid showing that the construct is capable of producing CRIP mRNA transcript.

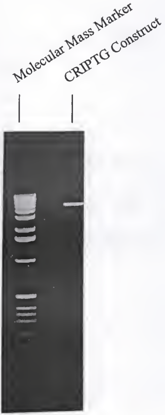

Figure 3-7: Photograph of ethidium bromide stained agarose gel of purified CRIPTG construct, verifying absence of contaminating DNA and establishing DNA concentration by comparison to DNA mass markers.

identification of the transgenic founders. It was not known whether CRIP overexpression would be lethal to the developing mouse or alter reproductive capability. Therefore, it was necessary to begin breeding as quickly as possible in an attempt to produce transgenic offspring which could be further studied.

Breeding F1 offspring to establish individual transgenic lines. Offspring from founder transgenic breeding pairs were analyzed for transgene integration using the polymerase chain reaction as described above for the identification of transgenic founders. An F1 female mouse (3626F1/11) was used to establish a transgenic line. Founder animals could be chimeric, meaning that the transgene was not integrated into the genome of every cell. Chimerism results when integration does not occur prior to division of the single-cell embryo. F1 offspring cannot, by definition, be chimeric, because either the oocyte or sperm from which it was produced contains the transgene integrated into its genome. In addition, founder animals may have the transgene integrated into more than one site in their genome. Typically, F1 mice from such founders inherit only one of the chromosomes with the transgene and subsequently have only one site of transgene integration in their genome. Therefore, littermates from founder mice may or may not be identical in terms of transgene integration site and number. In order to eliminate this variability, F1 offspring are typically used as colony founders. When bred with a non-transgenic mate, fifty percent of the offspring from an F1 transgenic mouse should be transgenic with the site of integration identical among all offspring. All transgenic offspring from such matings will be hemizygous for the transgene. Offspring from F1 generation mice are considered the F2 generation. In order to obtain homozygous mice, brother and sister F2 offspring are mated and their offspring are tested for transgene copy number to differentiate between hemi- and homozygosity.

Determination of CRIP Expression in Transgenic Mice

The presence of the transgene in the mouse genome does not guarantee expression of the gene. To determine whether the CRIPTG mice overexpressed CRIP, F1 or F2 offspring were sacrificed and analyzed for CRIP mRNA expression in various tissues. Evaluation of overexpression was accomplished by comparing CRIP mRNA levels in transgenic mice with that of a non-transgenic littermate. These comparisons were made for lines 3611, 3617, 3626 and 3635, as shown in figures 3-9 and 3-10. However, such comparisons were not made for line 3628 because no transgenic offspring were produced from this founder mouse. Because offspring from line 3626 showed the highest amount of overexpression in the most number of tissues, two F1 offspring, F1-9 and F1-11 were chosen to breed for establishment of the transgenic line.

Characterization of CRIPTG Mice - Line 3626

Determination of transgene copy number. Using slot blot analysis of 15 μg of genomic DNA from F0, F1 and F2 mice, the number of CRIPTG copies integrated into the genome of the 3626 line was estimated. Duplicate blots were probed with a CRIP or β-actin probe, autoradiographed and the density of each slot was determined as described in Chapter 2. A ratio of CRIP/Actin intensity was then determined and compared to a standard curve of known copy standards. These data are shown in Table 3-1. In all seven mice analyzed by slot blot, the copy number was estimated to be 5 copies/genome.

Tissue Survey of CRIP mRNA Expression. A survey of CRIP mRNA in various tissues from transgenic F1 mice demonstrated that CRIP overexpression was highest in the thymus, spleen, lung and heart of transgenic mice (figure 3-11). CRIP expression in the duodenum, jejunum and ileum of the transgenic small intestine appeared to be equivalent. Data for the duodenum are shown in figure 3-12.

Table 3-1: Slot Blot Data for Estimation of Transgene Copy Number

Mouse Number	**CRIP Intensity (Relative Densitometric Units)**	**Actin Intensity (Relative Densitometric Units)**	**Copy Number Estimate**
3626/F0	554	228	5.2
3626/F1-11	900	345	6.7
3626/F1-35	188	122	4.0
3626/F1-41	278	122	5.7
3626/F2-A42	670	292	5.9
3626/F2-A45	794	381	5.4
3626/F2-A47	722	322	5.7

CRIP mRNA expression was also analyzed in various tissues from F2 mice. A litter of mice was sacrificed and total RNA was extracted from the small intestine, thymus, heart, spleen, lung, kidney and liver of transgenic mice and their non-transgenic littermates. Northern analysis was performed for each tissue and membranes were hybridized with CRIP and 18S probes. Densitometric analysis was then performed and CRIP expression was normalized to 18S expression. Figures 3-12 through 3-16 show these data. Comparable to the F1 tissue survey, CRIP mRNA expression in the F2 tissues is greatest in the thymus, spleen and lung, overexpression in these tissues is significantly greater ($p<0.01$) for transgenic mice when compared to their non-transgenic littermates Although CRIP mRNA expression is increased in the liver and kidney of transgenic mice compared with their non-transgenic littermates, levels did not reach statistical significance in these tissues with the number of animals tested.

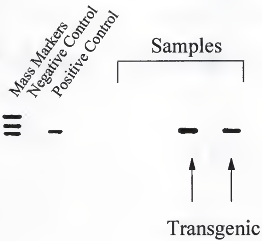

Figure 3-8: Identification of transgenic founder mice by PCR. DNA extracted from tails of weanling mice was analyzed by PCR using primers which would produce an 800 bp PCR product only if the transgene was present.

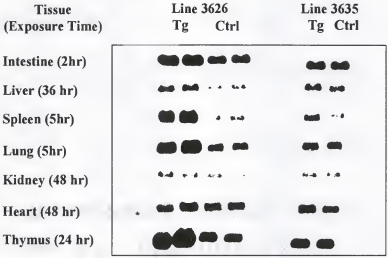

Figure 3-9: Northern analysis of CRIP mRNA expression in transgenic mouse lines 3626 and 3635. Total RNA from various tissues was extracted, electrophoresed (20 μg/lane) and transferred as described in Materials and Methods. Northern blot was probed with ^{32}P-rat CRIP probe. Equal loading of lanes was confirmed by ethidium bromide staining of RNA prior to transferring.

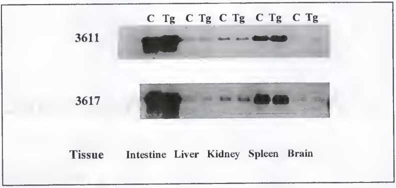

Figure 3-10: Northern analysis of CRIP transgenic mouse lines 3611 and 3617. Total RNA from various tissues was extracted, electrophoresed (20 μg/lane) and transferred as described in Materials and Methods. Northern blot was probed with ^{32}P-rat CRIP probe. Equal loading of lanes was confirmed by ethidium bromide staining of RNA prior to transferring.

Western analysis. Tissues from transgenic and control mice were compared using Western analysis to determine whether increased levels of CRIP mRNA also resulted in increased amounts of CRIP protein. Figures 3-17 and 3-18 show the results of the Western analyses performed on protein from small intestine, thymus and spleen.

ELISA. Intestinal mucosa homogenates prepared for Western analysis were also used in the CRIP ELISA (figure 3-19). CRIP protein levels were consistently higher in line 3626 transgenic mice (109.7 ± 22.3, mean ± SD) compared to their non-transgenic littermates (48.3 ± 20.6, mean ± SD, $p<0.05$). CRIP protein measured by ELISA in mice from line 3635 revealed an increase in CRIP in the transgenic vs. non-transgenic mouse, but less CRIP in the transgenic mouse from 3635 vs. transgenic mice from line 3626.

Immunohistochemistry. Immunohistochemistry was performed on tissue sections from normal and transgenic mice to determine whether morphological changes occurred in tissues where extra CRIP protein was produced, to examine localization of CRIP protein in the intestine and lymphoid organs of mice and to determine whether CRIP protein in organs from transgenic mice was localized similarly to endogenous CRIP protein. Figure 3-20 shows hematoxylin and eosin (H&E) stained intestinal sections (25X magnification) from a transgenic mouse and its non-transgenic littermate. There are no overt morphological changes between the two. Figure 3-21 shows intestinal sections (50X magnification) from the same animals, stained with an anti-CRIP IgG which produces a reddish-brown color and counterstained with hematoxylin. CRIP protein is seen in the mucosa, submucosa and lamina propria as well as in villus epithelial cells. Specificity of the anti-CRIP IgG used in these procedures is demonstrated in Figure 3-22, an excess of rhCRIP was incubated with the CRIP antibody overnight prior to its use as the primary antibody in the staining procedures. Very little background staining is seen, confirming the specificity of this antibody for CRIP.

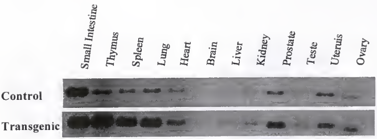

Figure 3-11: Tissue survey of CRIP RNA expression in CRIP transgenic mouse compared to non-transgenic littermate. Total RNA was extracted, electrophoresed (20 µg/lane) and transferred to a membrane as described in Materials and Methods. Northern blot was probed with ^{32}P-rat CRIP probe and exposed to film. Equal loading of lanes was confirmed by ethidium bromide staining of RNA prior to transfer.

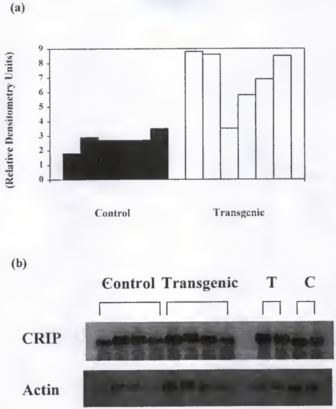

Figure 3-12: CRIP mRNA expression in duodenum of small intestine. (a). Histogram of densitometric quantitation of autoradiographs. Expression of CRIP (7.0 ± 2, mean ± SD) in intestine of transgenic mice is significantly increased ($p<0.01$) compared to CRIP expression (2.7 ± 0.6) in intestine of normal mice. CRIP mRNA levels were normalized to β-actin mRNA expression. (b). Northern blots of intestine total RNA (20 μg/lane) probed with ^{32}P-rat CRIP and ^{32}P-mouse β-actin probes.

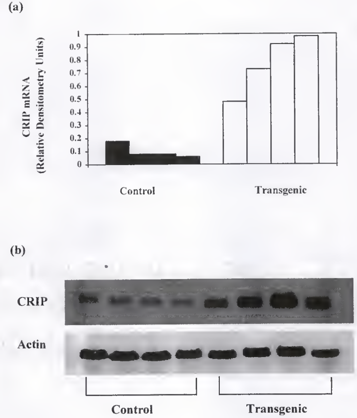

Figure 3-13: CRIP mRNA expression in thymus. (a). Histogram of densitometric quantitation of autoradiographs. Expression of CRIP (0.7 ± 0.2, mean ± SD) in thymus of transgenic mice is significantly increased ($p<0.001$) compared to CRIP expression (0.10 ± 0.05) in thymus of normal mice. CRIP mRNA levels were normalized to β-actin mRNA expression. (b). Northern blots of thymus total RNA (20 μg/lane) probed with ^{32}P-rat CRIP and ^{32}P-mouse β-actin probes.

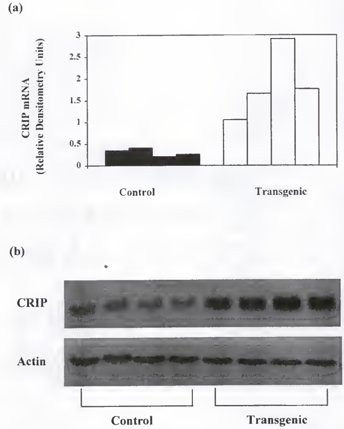

Figure 3-14: CRIP mRNA expression in spleen. (a). Histogram of densitometric quantitation of autoradiographs. Expression of CRIP (1.9 ± 0.8, mean ± SD) in spleen of transgenic mice is significantly increased ($p<0.01$) compared to CRIP expression (0.3 ± 0.1) in spleen of normal mice. CRIP mRNA levels were normalized to β-actin mRNA expression. (b). Northern blots of spleen total RNA (20 μg/lane) probed with ^{32}P-rat CRIP and ^{32}P-mouse β-actin probes.

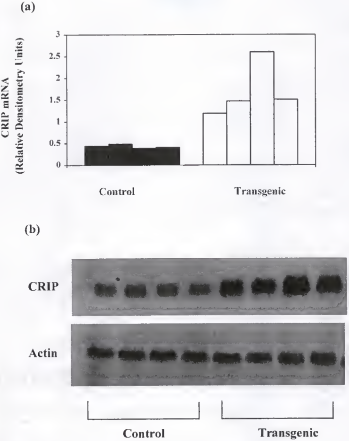

Figure 3-15: CRIP mRNA expression in lung. (a). Histogram of densitometric quantitation of autoradiographs. Expression of CRIP (1.7 ± 0.6, mean ± SD) in lung of transgenic mice is significantly increased ($p<0.007$) compared to CRIP expression (0.43 ± 0.04) in lung of normal mice. CRIP mRNA levels were normalized to β-actin mRNA expression. (b). Northern blots of lung total RNA (20 μg/lane) probed with ^{32}P-rat CRIP and ^{32}P-mouse β-actin probes.

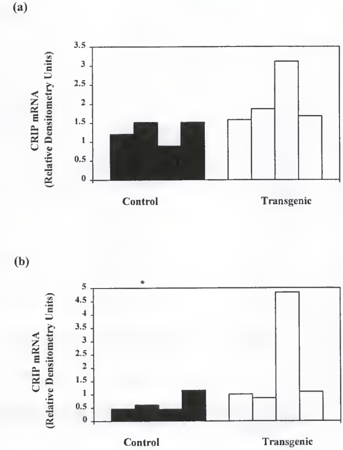

Figure 3-16: Histogram of densitometric quantitation of autoradiographs showing CRIP mRNA expression in liver and kidney. (a). CRIP expression in liver of transgenic mice (2.1 ± 0.7, mean ± SD) was not significantly increased ($p<0.09$) compared to CRIP expression (1.3 ± 0.3) in liver of normal mice. (b). CRIP expression in kidney of transgenic vs. non-transgenic mice was not significantly increased (2.0 ± 2 vs. 0.7 ± 0.3, $p<0.24$).

CRIP protein was also localized in mouse lymphoid organs. Figure 3-23 shows H&E stained thymus from a transgenic mouse and its non-transgenic littermate. The darker stained, outer section of the organ is the thymic cortex and the inner, lighter stained compartment is the medulla. A hallmark of the medulla, a structure known as a Hassal's corpuscle is noted by the arrow in the medulla of each section. Note in figure 3-24 the absence of CRIP staining in the Hassal's corpuscle, suggesting that these structures do not contain CRIP protein. Also of note in the thymus is the intense CRIP staining in the medulla as compared to the cortex. CRIP protein has also been localized in the popliteal lymph node. As is shown in figure 3-25, CRIP protein appears to be present in the outer cortex region of the lymph node in both transgenic and non-transgenic tissues. Slightly more CRIP may be present in the node of the transgenic mouse. CRIP protein was also noted in the marginal zones of the white pulp in spleen from transgenic and non-transgenic mice (data not shown). Our lab has previously shown that peritoneal macrophages obtained from rats express CRIP mRNA in abundance. Figure 3-26 demonstrates that mouse peritoneal macrophages produce CRIP protein as well and that this protein appears to be localized in the cytoplasm of these cells.

Flow cytometry. The high levels of CRIP mRNA and protein noted in the thymus, an organ primarily responsible for development of immunocompetent T lymphocytes led to the hypothesis that CRIP may play a role in the proliferation or maturation of these cells. To determine whether extra CRIP protein in the thymus would alter the population

of thymic lymphocytes, flow cytometry was performed on single-cell suspensions prepared from thymus of transgenic mice and their non-transgenic littermates. Figure 3-27 shows a scatter diagram of unstained thymocytes depicting similar populations of thymocytes from non-transgenic and transgenic mice, and figure 3-28 shows the contour plots of thymotcytes stained with labeled CD4 and CD8 antibodies. The results of the

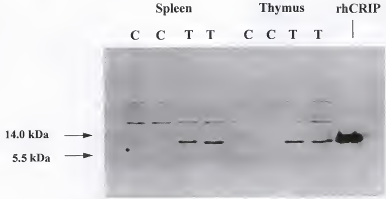

Figure 3-17: Western analysis of CRIP protein in thymus and spleen. Total cytosolic protein (40μg/lane) was separated using 15% SDS-PAGE, transferred to nitrocellulose and incubated with anti-CRIP IgG. ECL^{TM} was used to detect labeled protein. Recombinant human CRIP (rhCRIP) was used as a control.

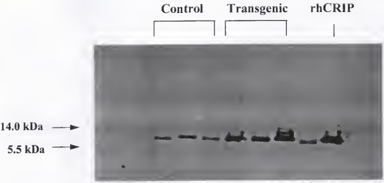

Figure 3-18: Western analysis of CRIP protein in small intestine. Total cytosolic protein (40μg/lane) was separated using 15% SDS-PAGE, transferred to nitrocellulose and incubated with anti-CRIP IgG. ECL^{TM} was used to detect labeled protein. Recombinant human CRIP (rhCRIP) was used as a control.

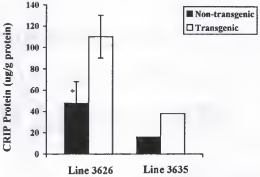

Figure 3-19: Comparison of CRIP protein in intestinal mucosa of transgenic vs. non-transgenic mice from lines 3626 (109.7 ± 22.3 vs. 48.3 ± 20.6 µg/g protein, mean + SD $p < 0.05$, n=3)and 3635 (38.2 vs. 15.9, n=1).

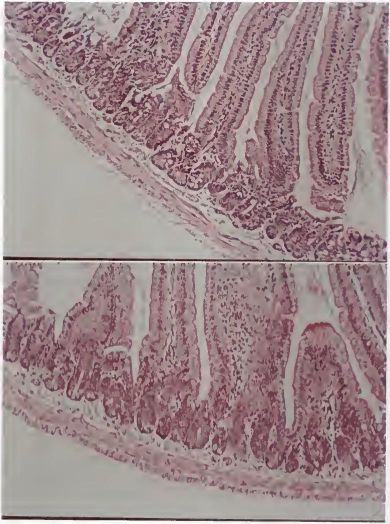

Figure 3-20: Hematoxylin and eosin staining of small intestine from non-transgenic (top) and transgenic (bottom) mice (50X magnification).

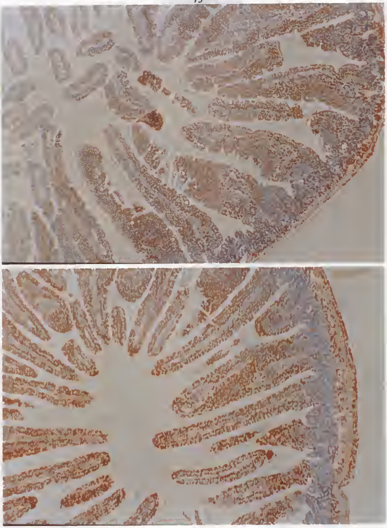

Figure 3-21: Small intestinal sections from non-transgenic (top) and transgenic (bottom) mice showing localization of CRIP (reddish-brown stain, 25X).

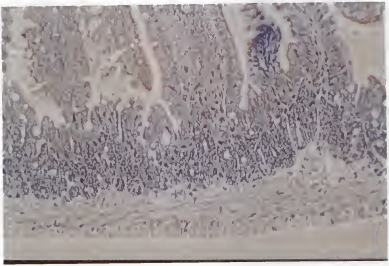

Figure 3-22: Small intestinal section from non-transgenic mouse showing specificity of antibody for CRIP protein (50X).

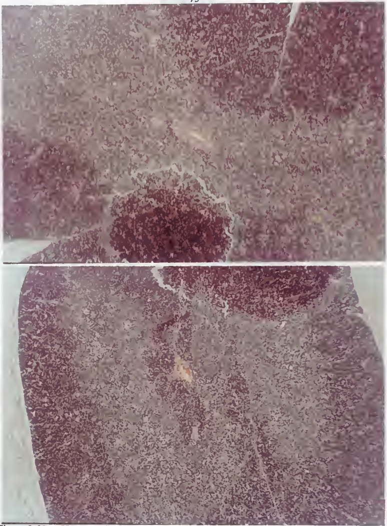

Figure 3-23: Hematoxylin and eosin staining of thymus sections from non-transgenic (top) and transgenic (bottom) mice (25X).

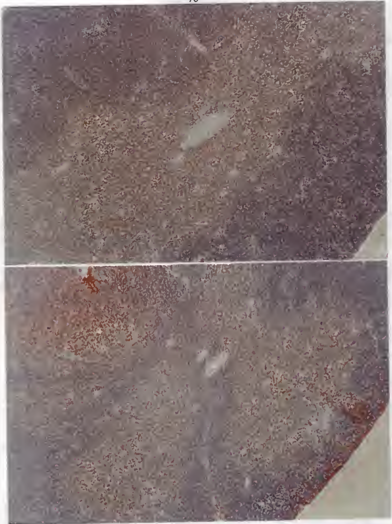

Figure 3-24: Thymus sections from non-transgenic (top) and transgenic (bottom) mice showing localization of CRIP protein in thymic medulla (25X).

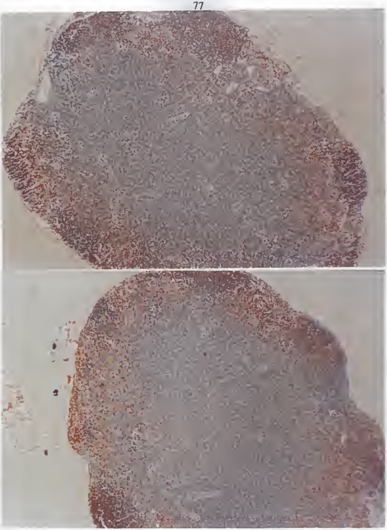

Figure 3-25 Lymph node sections from non-transgenic (top) and transgenic (bottom) mice showing localization of CRIP protein in outer cortex (25X).

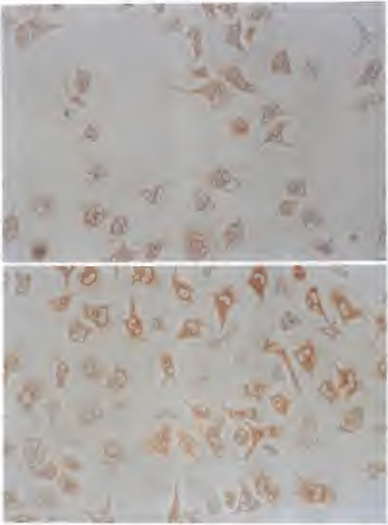

Figure 3-26: Peritoneal macrophages from non-transgenic (top) and transgenic (bottom) mice showing localization of CRIP protein in cytoplasm (100X).

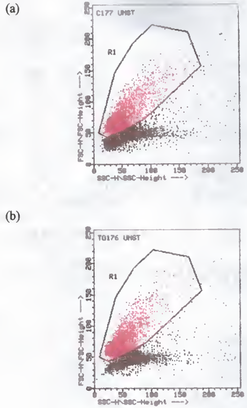

Figure 3-27: Scatter diagram of unstained thymocyte cell populations based on size and granularity showing similar distribution of cells from non-transgenic (a) and transgenic (b) mice. Gated cells represent thymic lymphocyte population.

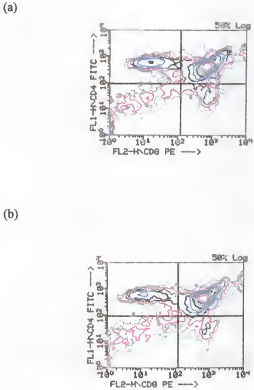

Figure 3-28: Contour plot demonstrating CD4 (y-axis) and CD8 (x-axis) thymocyte populations in mice. (a). Thymocytes from non-transgenic mouse. (b). Thymocytes from transgenic mouse.

flow cytometric analyses summarized in Table 3-2 indicate that there is a higher percentage of immature $CD4^+/CD8^+$ lymphocytes in thymus from transgenic mice compared to their non-transgenic littermates.

Miscellaneous analyses. Body mass was determined in animals sacrificed for experimental purposes. No significant difference between body mass (mean ± SD) in transgenic (20 ± 5 g) vs. non-transgenic (21 ± 5 g) littermates was noted. Serum zinc concentration (mean ± SD) was also similar between transgenic mice and their non-transgenic littermates (1.2 ± 0.1 vs. 1.1 ± 0.1 µg/mL, respectively). In addition, blood chemistry profiles including total protein, albumin:globulin ratio and complete blood count were also performed on blood collected from transgenic mice and their non-transgenic littermates. All values were within normal limits.

Table 3-2: Flow Cytometry Analysis of Thymic Lymphocyte Populations

	$CD4^+/CD8^+$	$CD4^+/CD8^-$	$CD4^-/CD8^+$
Experiment #1			
Control	77.9 ± 0.5	13.9 ± 0.5	3.5 ± 0.2
Transgenic	82.0 ± 0.2[a]	10.9 ± 0.2[b]	2.7 ± 0.1[b]
Experiment #2			
Control	67.5 ± 0.3	21.4 ± 0.2	5.2 ± 0.2
Transgenic	77.7 ± 0.7[a]	14.8 ± 0.2[a]	3.5 ± 0.3[a]

a = $p \leq 0.01$, b = $p \leq 0.05$ for control vs. transgenic within experiment

CHAPTER 4
DISCUSSION, SPECULATIONS AND CONCLUSIONS

Discussion and Speculations

The discovery of cysteine-rich intestinal protein by Birkenmeier and Gordon in 1986 identified the existence of a novel protein produced in abundance in the intestine. Subsequent investigations have provided information about the structure and tissue distribution of CRIP and have also provided information regarding the regulation of CRIP expression in various rat tissues during development and following immune (LPS) and toxic (CCl_4) challenges. The research described in this dissertation compliments existing information, providing data about the genomic organization of CRIP, its tissue distribution in mice, localization of CRIP protein in lymphoid tissue and the effect of CRIP overexpression on its tissue localization as well as on the thymic lymphocyte population.

Levenson et al. (1994a, 1994b) sequenced the CRIP promoter and examined regulatory elements contained within this region. They documented the lack of a TATA box but the existence of a GC-rich region on the promoter capable of binding the general transcription factor, Sp1. Glucocorticoid regulation was also demonstrated in intestine from neonatal rats and in transfected IEC-6 cells. The organization of the CRIP promoter/regulatory region suggested a role for CRIP in a cellular housekeeping capacity.

In this dissertation project, the rat CRIP structural gene was sequenced which identified its genomic organization (Davis et al., 1996). Five exons, disrupted by four introns, contain the sequence information for rat CRIP. Interestingly, the double zinc finger, LIM motif, is not coded for within one intron. This is consistent with the other LIM proteins that have been sequenced, CRP, rhombotin and mec-3, all of which have their LIM motifs disrupted by one or more introns. Since the LIM motif is a sequence that is repeated 2-5 times in many LIM proteins, it was tempting to speculate that the single LIM domain of CRIP might be coded for in one exon, acting as a cassette that was duplicated in the genes of proteins which contained more than one LIM motif. The presence of the LIM motif in a growing number of proteins coupled with the disruption of the LIM coding sequence by several introns, suggests that CRIP and the LIM domain are sequences that have been subjected to genetic recombinations resulting in their current genomic organization.

The presence of a glucocorticoid response elements (GRE) in intron 1 is interesting since several GREs were previously found in the CRIP promoter and CRIP expression appears to be modulated by glucocorticoids in the neonatal intestine just prior to weaning (Levenson et al., 1993). Perhaps the significance of glucocorticoid regulation is related to the function of CRIP during the neonatal period. The existence of so many GREs within the CRIP gene suggests that they play an important role in regulating CRIP expression. It is logical, therefore, that this could be related to the importance of CRIP expression during development. Glucocorticoids are known to increase digestive enzyme production by the intestine during the neonatal period (Needleman et al., 1993). How that relates to CRIP expression is not clear but fits with the notion of a role in differentiation. Also, glucocorticoids are known suppressers of immune response and inflammation (Caster et al., 1993; Janeway and Travers, 1996). CRIP has previously been shown to be upregulated late in the immune response, during a period when suppression of the immune response would occur. It will be important to determine whether CRIP expression is sensitive to glucocorticoid regulation in immune tissues and whether glucocorticoids modulate CRIP expression during an immune response.

The presence of a B1 repetitive element within intron one of the CRIP gene is evidence that such recombination events could be responsible for part of the genomic organization of CRIP. The B1 repetitive element is one of several rodent short interspersed repetitive DNA sequences that often is inserted into new genomic sites by the copying of RNA molecules into DNA in a process known as retroposition. The existence of certain structural features may favor insertion of repetitive sequences. Repeated DNA sequence families in rodents and other mammals are thought to be 60-85 million years old (Deininger and Daniels, 1986).

Sequencing the rat CRIP structural gene enabled the design of PCR primers for production of the CRIP transgene and subsequent generation of transgenic mice. The number of live mice born and founder animals identified from the microinjected embryos were typical of the yields expected for generation of transgenic mice via the microinjection procedure (Overbeek, 1994). Expression from the transgene is often independent of the number of copies integrated into the genome. As few as one copy to as many as several hundreds of copies of the transgene may integrate, usually in a tandem head-to-tail fashion. Although the mechanisms responsible for integration are being investigated, the molecular events leading to integration are currently unknown (Brinster et al., 1981). Transgene expression is dependent upon the site of integration, where surrounding regulatory elements or genomic (chromatin) structure can regulate the activity of transcriptional machinery and therefore influence transcription from the transgene. These factors could be responsible for the varying levels of CRIP expression noted for the transgenic mice described in this project. For example, very little overexpression was noted in RNA extracted from tissues of animals from lines 3611, 3617 and 3635, whereas line 3626 consistently produced more CRIP RNA and protein. Animals from line 3626 had 5 copies of the CRIP transgene integrated into their genome, whereas animals from the other lines had only 1 or 2 extra copies. It is unknown whether the higher copy number or site of transgene integration has a greater influence on the production of CRIP in line 3626.

In order to provide an overexpression model for the study of normal CRIP function, the rat CRIP promoter was chosen to drive expression from the CRIP transgene construct. It was hoped that by using this promoter, CRIP expression would be localized in the same tissues in which it was normally produced. By both RNA tissue survey and immunohistochemical techniques it was shown that CRIP expression in transgenic mice was similar to expression in non-transgenic mice. Interestingly, expression in the thymus, spleen and lungs was increased considerably in transgenic vs. non-transgenic mice. Although increased levels of CRIP mRNA and protein were noted in the small intestine as well, the magnitude of difference between transgenic and non-transgenic mice in this tissue was not as dramatic as in the lymphoid organs and lungs. It may be possible that there is a threshold below which CRIP levels are maintained and that the endogenous production of CRIP in the small intestine is closer to this level, thereby limiting the extent to which overexpression can occur in the transgenic intestine.

Despite the dramatic increase in CRIP mRNA and protein in organs which normally produce CRIP in relatively high abundance, tissues which normally produce modest amounts of CRIP show only slightly elevated expression in transgenic vs. non-transgenic mice. For example, hybridization of brain, liver and kidney CRIP mRNA from normal mice is barely visible on an overnight exposure of a ^{32}P-labeled Northern blot, whereas a similar exposure of small intestine, thymus, spleen, lung or mononuclear cells produces an overexposed autoradiograph. This same relative intensity in CRIP expression between tissues was also observed in the transgenic mice. Thus, use of the endogenous CRIP promoter allowed CRIP expression from the transgene to occur in a tissue-specific manner and in a similar relative intensity among tissues as is seen in non-transgenic mice. Therefore, use of these mice in experiments designed to compare the effects of CRIP overexpresssion in transgenic vs. non-transgenic mice will be appropriate because CRIP expression is occurring in a similar fashion. It does not appear that ectopic CRIP expression is occurring since no CRIP message was found in tissues which normally do not produce CRIP, i.e., adipose, pancreas and skeletal muscle (data not shown).

The immunohistochemical localization of CRIP protein in transgenic and non-transgenic small intestine, thymus, spleen, lymph node and peritoneal macrophage has provided important information about the distribution of CRIP protein in these tissues. It is not surprising to find that a protein produced in abundance in the small intestine is also produced abundantly in other lymphoid tissue. Afterall, gut-associated lymphoid tissue (GALT) comprises 80% of the body's lymphoid tissue (Castro and Arntzen, 1993). CRIP has previously been implicated in development/maturation of the gut due to its developmental regulation in this tissue. It is interesting to speculate that CRIP could be involved in the development and maturation of the mucosal defense system in this organ as well. Temporally, CRIP expression coincides with the upregulation in T cell numbers and the development of germinal centers within the lymphoid follicles and lamina propria of the intestine which occurs following birth when the gut is exposed to dietary and bacterial antigens (Shanahan, 1994). In the mouse, CRIP protein was found in the lamina propria and in the epithelial cells of the villus. CRIP staining was also seen in the submucosa and muscularis mucosa. Although CRIP protein has been localized to the Paneth cells of rat small intestine, only a few mouse Paneth cells appeared to express CRIP protein. There was no difference in the localization of CRIP protein in non-transgenic vs. transgenic mouse small intestine, suggesting that the lack of Paneth cell CRIP in mouse small intestine compared to rat may be a species-specific phenomenon. The mice used in this project were raised under SPF conditions. This may limit colonization by specific microflora which has recently been shown to influence intestinal differentiation (Bry et al., 1996). A comparison of CRIP localization in transgenic mice reared under SPF vs. normal conditions would provide insight as to whether bacterial colonization influences gut development in the presence of high levels of CRIP protein.

The presence of CRIP in the lamina propria does support a biological role for CRIP in mucosal defense as B and T cells as well as macrophages populate this area of the intestine and could account for some of the CRIP staining seen there. In fact, evidence for a close relationship between mucosal T cells and enterocyte proliferation and maturation has recently been documented showing that depletion of activated regulatory T cells in the lamina propria may lead to a breakdown of the mucosal barrier which in turn causes mucosal atrophy and enterocyte dysfunction (Zeitz et al., 1994). It is obvious that maintaining mucosal integrity is essential for optimal performance of absorptive and mucosal barrier functions in the intestine. It is possible that substances such as CRIP, present in both immune cells and intestinal epithelial cells may be involved in maintaining this barrier.

The abundance of CRIP in villar epithelial cells is noteworthy because of the distinct appearance of CRIP staining in the epithelial cells as they pass the crypt-villus junction to begin migration up the villus. These epithelial cells mature and differentiate as they migrate upward as compared to the intestinal epithelial cell population in the intestinal crypts which are stem cells that proliferate to replace the entire intestinal epithelium every 4-6 d. Future work with the CRIP transgenic mice should include investigation of the maturational state of villar epithelial cells in response to extra CRIP production. For example, it would be interesting to determine whether the appearance of markers of intestinal maturation such as sucrase-isomaltase or alkaline phosphatase is altered in transgenic mice. If CRIP is involved in the intestinal maturation process, the normal turnover of villar epithelial cells may be altered in transgenic vs. non-transgenic animals as well.

CRIP mRNA and protein were also localized to the thymus of both non-transgenic and transgenic mice. The thymus is composed of both epithelial and immune cells. Thymocytes proliferate in the outer cortex of the thymus and migrate toward the inner medulla area as they mature into T lymphocytes and undergo selection processes. CRIP protein was found to be concentrated within cells of the thymic medulla, suggesting that more mature cells make this protein. This is in agreement with the presence of CRIP in more mature epithelial cells in the intestine. It cannot be ruled out, however, that the CRIP present in the thymus originates in the medullary epithelial cells to create a microenvironment which fosters the maturation of T cells undergoing differentiation and selection processes. In order to differentiate between these two alternatives, further localization studies need to be performed. Specifically, double-labeling techniques could be utilized to determine whether lymphocytes expressing cell-specific surface markers also express CRIP. This would help distinguish between CRIP expression in mature vs. immature lymphocytes as well as distinguish between CRIP expression from thymic epithelial vs. T cells.

From flow cytometry analysis of lymphocyte populations in transgenic vs. non-transgenic mice, it appears that the presence of extra CRIP protein may influence T cell development within the thymus. Elevated levels of CRIP mRNA and protein were present in thymus from transgenic mice and these mice exhibited altered populations of lymphocytes expressing $CD4^+/CD8^+$, $CD4^+/CD8^-$ and $CD4^-/CD8^+$ surface markers as assessed by flow cytometry. Transgenic mice overexpressing CRIP protein in the thymus had a larger proportion of less mature double-positive ($CD4^+/CD8^+$) thymocytes as compared to their non-transgenic littermates. Accordingly, the proportion of single-positive thymocytes from the transgenic mice was decreased compared to those from non-transgenic littermates. This may indicate that CRIP is involved in control of cellular differentiation, since there are more immature cells and less mature cells in response to CRIP overproduction. Furthermore, as described above, the localization of CRIP protein in the thymic medulla supports a function for CRIP in cellular maturation or differentiation. These findings are consistent with the functions of other LIM-only proteins, such as Ttg-1, a transcription factor shown to be involved in cell proliferation (McGuire et al., 1992) and MLP, which is involved in muscle cell differentiation (Arber et al., 1994).

Sensitivity of immature thymocytes to zinc has been previously documented. Telford and Fraker (1995) demonstrated increased zinc-induced apoptosis in a less mature double-positive ($CD4^+/CD8^+$) thymocyte subset compared to death occurring in more mature subsets. Zinc deficiency has been shown to alter subsets of T lymphocyte populations (Gershwin et al., 1991), cause thymic atrophy, decreased natural killer cell activity and T lymphocyte responsiveness (Keen and Gershwin, 1990). Therefore, it is not surprising to find that a zinc-containing protein produced in abundance in the thymus, a lymphoid organ responsible for T lymphocyte development, could be involved in controlling this process. The ability of CRIP to bind zinc in vivo (Hempe and Cousins, 1991; Khoo and Cousins, 1994) and in vitro (Kosa et al., 1994) may relate to this effect of dietary zinc intake on the thymus.

One caveat to these conclusions is the fact that the histology and flow cytometry analyses described here were performed in mice from a single transgenic line, 3626.

Because integration of the transgene occurs at a random, unknown location in the genome, there is the possibility that a gene essential for lymphocyte differentiation could have been disrupted leading to the alterations noted in the thymic lymphocyte populations of CRIP transgenic mice. Arguing against an effect due to an insertional mutation caused by transgene integration is the high expression of CRIP in lymphoid tissue, its regulation by immune challenge and membership in a protein family known to be involved in differentiation and proliferation of immune cells. A role for CRIP in regulation of immune cell maturation, based upon the results with CRIP transgenic mice described here, is consistent with what is currently known about this protein (e.g. regulation by immune challenge and abundance in immune cells). Also, it would be expected that insertional disruption of a gene important in T cell maturation would lead to much more dramatic alterations in thymocyte populations than have been witnessed in these transgenic mice.

We chose to expand the 3626 line of transgenic mice because CRIP expression in this line was highest relative to non-transgenic mice and mice from other lines. Since the 3635 founder animal is still alive, it will be possible to produce offspring that can be tested to determine whether CRIP overexpression alters thymic lymphocyte populations. However, this line of mice does not overexpress CRIP to the level that mice from the 3626 line do. Therefore, if there is no change (or a statistically insignificant change) in lymphocyte populations in mice from this line, it will not be possible to distinguish between a gene dose effect or a positional effect. For example, since the 3635 line does not produce as much CRIP as the 3626 line, there would not be as much CRIP protein in the thymus of mice from line 3635 as compared to 3626. Since, the effect of CRIP on the lymphocyte population was only modest in response to very high CRIP levels in the 3626 line, the lesser amount of CRIP in the 3635 line may not be enough to reproduce the response seen in line 3626. If this occurred, it would not be possible to determine whether it was due to the fact that transgene integration was in a different location in 3635 vs. 3626 mice, or whether it was due to the difference in CRIP production between these two lines. Therefore testing the 3635 line may be of limited value. The other option would be to develop another group of founder animals and screen the progeny of these for characteristics found to be altered in the 3626 mouse line.

CRIP localization in the spleen and lymph node, two peripheral lymphoid organs, underscores the importance of this protein in immune tissues and justifies further investigation in this area. Of particular significance would be to determine whether CRIP expression is developmentally regulated in immune tissues, especially the thymus. In addition, identification of CRIP expression in bone marrow would also be an important area of investigation. Since thymocytes originate in the bone marrow, it would be interesting to determine whether these very immature stem cells express CRIP. This may help to distinguish between a role for CRIP in proliferation vs. differentiation within this cell type.

CRIP transgenic mice will provide an ideal model to explore the function of this protein in lymphoid tissue. Immune challenge experiments can be designed to determine whether increased CRIP expression alters the immune response. Lymphocyte proliferation assays, employing specific T and B cell mitogens, will enable comparisons between the effect of CRIP overexpression on different populations of lymphocytes following immune challenge. This mouse model will also aid in future investigations aimed at determining whether CRIP is involved in intestinal maturation or mucosal immunity. Finally, although the presence of CRIP in reproductive tissue was not studied in detail for this project, CRIP overexpression was noted in reproductive tissue in transgenic mice. Therefore, these mice may also serve as a helpful model for identifying a potential function CRIP in reproductive organs as well.

Conclusions

This dissertation project utilized several tools of molecular biology to further study the zinc-binding protein, cysteine-rich intestinal protein. The rat CRIP structural gene was sequenced which provided information about the organization of this gene. From this information, a transgenic mouse model was developed and characterized for the further study CRIP. Overexpression of CRIP in these mice was shown to follow a pattern of tissue distribution similar to endogenous CRIP mRNA and protein. The distribution of CRIP protein within the intestine and lymphoid tissue also followed a pattern similar to endogenous CRIP expression, as assessed by immunohistochemical techniques. Initial analyses with these mice suggest that CRIP may be involved in control of cellular proliferation or differentiation. These transgenic mice will provide a useful model for further investigation of the function of CRIP in these cellular processes. They represent a significant contribution to the study of this protein's function by providing a means for comparing the effects of CRIP overexpression in otherwise normal mice. Because of their relative abundance, zinc-finger proteins are thought to comprise a significant proportion of the dietary zinc requirement. For this reason, it is nutritionally significant to study these proteins in order to enhance our understanding of the metabolism of zinc at a molecular level. Such investigation may uncover important biomarkers for zinc status as well as aid in more accurate estimation of human zinc requirements.

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BIOGRAPHICAL SKETCH

Barbara Ann Davis was born in Torrington, CT, on 25 April 1967 to Diane G. Carlson and Robert A. Moreschi, Sr. She graduated from Torrington High School in 1985 and attended St. Joseph College, West Hartford, CT, where she received her bachelor's degree in foods and nutrition in 1989. She entered graduate school in 1989 and working under the direction of Nancy F. Sheard, Sc.D., R.D., received her master's degree in nutrition science in 1991 from the University of Massachusetts, Amherst. While working on her M.S. degree at the University of Massachusetts, Barbara also completed the Approved Pre-professional Practice Program in Dietetics and became a registered dietitian in 1991. Following graduation from the University of Massachusetts, where she was awarded the Helen Mitchell Scholarship and a Ross Laboratories Scholarship, she worked at Bay State Medical Center in Springfield, MA, as a registered dietitian.

In 1992, Barbara began graduate studies at the University of Florida in the Food Science and Human Nutrition Department. In 1993, she married Paul E. Davis and in 1995, she was inducted into the Sigma Xi, National Research Honor Society. After completion of her doctorate, Barbara and Paul along with their cat, two black labs and parrot will move to Blacksburg, VA, where Barbara will begin a tenure-track faculty appointment at Virginia Polytechnic Institute and State University in the Human Nutrition, Foods and Exercise Department.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Robert J. Cousins, Chair
Boston Family Professor of Nutrition

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Jesse F. Gregory

Jesse F. Gregory, III
Professor of Food Science and
Human Nutrition

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Bobbi Langkamp-Henken

Bobbi Langkamp-Henken
Assistant Professor of Food Science
and Human Nutrition

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Susan S Percival

Susan S. Percival
Associate Professor of Food Science
and Human Nutrition

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Sheldon M. Schuster
Professor of Biochemistry and
Molecular Biology

This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December, 1996

Jack L. Fry

Dean, College of Agriculture

Dean, Graduate School

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